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Inhibition of *in vitro* natural killer activity by the third component of complement: Role for the C3a fragment

(spontaneous cytotoxicity/regulation/complement/structure–function)

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ABSTRACT **Purified human native third component of complement, C3, was found to inhibit *in vitro* natural killer (NK) cell cytotoxicity in both mouse and human systems. The effect was dose and time dependent, a 50% inhibition being reached with 190 nM C3 (35 μg/ml) added during the NK assay or after a 30-min preincubation of the effector cells with this C3 concentration. C3 was shown to act at the effector-cell population level because pretreatment of the target cells did not modify the NK lysis. The inhibition was not due to general cytotoxicity nor to cell agglutination. Moreover, another *in vitro* cytotoxicity system (represented by alloreactive cytotoxic lymphocytes) was not affected by purified C3. Structural analysis of the active part of the C3 molecule shows that the C3-induced inhibition is supported by the C3a fragment. Release of carboxyl-terminal arginine residue by carboxypeptidase B, converting C3a into des-Arg^{77}-C3a, did not alter the inhibitory effect displayed by this fragment. These results suggest that C3a may play an important role in the regulation of NK activity.**

Natural killer (NK) cells are defined as spontaneously cytotoxic lymphocytes with broad target specificity mainly directed against tumor cells and virus-infected cell lines (1, 2), but the mechanisms controlling their cytotoxicity level are still poorly understood. In this regard, it has been shown that NK cell cytotoxicity can be strongly increased by interferons (IFNs) released during a viral infection (IFN-α) (3) or during the activation of T cells (IFN-γ) (4) and also by interleukin-2 (5). On the other hand, macrophages from mice injected with polyanions have been reported to exert suppressive effects upon NK activity (6, 7). Noteworthy findings are that macrophages are well known to express receptors for the native third component of complement, C3, on their cell membrane (8) and also to synthesize C3 molecules (9). Moreover, bacteria and polyanions are activators of the alternative pathway of the complement in which C3 is the pivotal protein and a precursor of several fragments (10). Some of the C3 fragments have properties enabling reaction with cell surfaces through either acceptor sites (as for C3b) (11) or receptor sites (as for C3a, C3b, iC3b, and C3d) (8–12), and various biological functions have been associated with these fragments. Thus, C3a is an anaphylatoxin (13), and C3b (or iC3b and C3d) can mediate attachment of target particles and soluble complexes to macrophages and to B cells that express specific membrane receptors for these fragments, promoting in this way their specialized cell functions (14).

Such data prompted us to investigate the possible functional relationship between C3 and NK activity. In this report, we show that highly purified human C3 has an inhibitory effect on *in vitro* mouse and human NK activities. This inhibition is dose and time dependent, acts at the effector-cell population level, and is carried by a small fragment of C3, the C3a.

MATERIALS AND METHODS

Effector Cells. Mouse spleen cells were harvested from 2-month-old *nude* C57BL/6 mice bred in the Institut de Recherches Scientifique sur le Cancer animal facilities. In some experiments they were run through nylon wool columns to remove B lymphocytes and macrophages (15). Extensive elimination of macrophages also was achieved by *in vivo* intravenous injection of 2 mg of silica per mouse (quartz, <5 μm; Dörentrup, Mannheim, Federal Republic of Germany), followed by filtration through Sephadex G-10 columns. In some other cases, removal of erythrocytes was achieved by hypotonic shock with distilled water. Human NK effector cells were prepared from the leukocyte-enriched buffy coats of normal blood donors obtained through the courtesy of the blood Center of the Hopital Paul Brousse (Villejuif, France). Mononuclear cells were isolated by Ficoll/Isopaque (density, 1.078) gradient centrifugation (16), briefly treated with distilled water to remove erythrocytes, and then run through nylon columns. After an overnight incubation at 37°C in Falcon culture flasks to eliminate the remaining adherent cells, the mononuclear cells were fractionated on discontinuous Percoll density gradients as described by Timonen and Saksela (17). Low-density fractions in which peak NK activity was detected and which were strongly enriched for large granular lymphocytes (LGL) were collected and used throughout our tests.

Target Cells. YAC-1, a Moloney virus-induced lymphoma in A/Sn mouse, and K 562, a human myeloid cell line, were used for measurement of murine and human NK activities, respectively.

Culture and Test Media. RPMI 1640 medium (GIBCO) containing 10% fetal calf serum (GIBCO), 100 units of penicillin per ml, and 100 μg of streptomycin per ml was used.

Cytotoxicity Assay. Target cells (10^7 YAC-1 or K 562) were suspended in 1 ml of serum-free medium and labeled with 150 μCi of $Na_2\,^{51}CrO_4$ [specific activity, 166 mCi/mg (614 GBq/mg); Commissariat a l'Energie Atomique, Saclay, France] for 1 hr at 37°C. They were then washed twice and adjusted to 2 × 10^6 cells per ml in culture medium. ^{51}Cr-Labeled target cells (0.1 ml) were mixed with 0.1 ml of spleen cells in each well of 6-mm flat-bottomed Falcon TC 3040 microtest plates at an ef-

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Abbreviations: LGL, large granular lymphocytes; E/T, effector-cell/target-cell ratio; C3, native third component of complement; NK cells, natural killer cells; IFN, interferon.

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fector-cell/target-cell (E/T) ratio varying from 100:1 to 12:1. Cultures were set up in triplicate and incubated for 4 hr at 37°C in humidified 5% CO_2/95% air. At the end of the incubation period, 100 μl of the supernates were transferred into tubes and counted in a multichannel gamma spectrometer (Multigamma; LKB). The percentage of specific ^{51}Cr release was determined according to the formula $(E - S/T - S) \times 100$ in which E is the ^{51}Cr release in the presence of the effector spleen cells, S is the spontaneous ^{51}Cr release, and T is the total release in the presence of 2 M HCl.

Purification of Human C3. Human C3 was purified following its hemolytic activity as described by Tack and Prahl (18). Homogeneity was assessed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (Fig. 1*A*, lane 1) and by immunoelectrophoresis with rabbit anti-C3 antiserum prepared in our laboratory or with goat anti-C3 antiserum (Miles, Slough Bucks, England). The C3 preparations were free of C5, factor B, factor D, properdin, and H activity.

Cleavage of Human C3 and Purification of the C3a Fragment. Purified C3 (1 mg/ml) in phosphate-buffered saline was treated with 0.2% trypsin for either 3 min or 30 min at 37°C. C3 cleavage was terminated by addition of 0.4% soybean trypsin inhibitor. When C3 was treated for only 3 min, C3a (Fig. 1*B*, lane 1) and C3b (Fig. 1*A*, lane 2) were generated, whereas during a 30-min treatment, the C3b fragment was in turn mainly converted into C3c and C3d, and the C3a fragment was altered both structurally and functionally as previously shown (19). After cleavage, 1.5 M NaCl was added to the C3a and C3b mixture as described by Tack *et al.* (20). C3a was separated from C3b by filtration on PM 30 membrane (Diaflo, Amicon) at 4°C. C3a and C3b were dialyzed against phosphate-buffered saline, and the cleavage efficiency was controlled on $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*A*).

Preparation of Human des-Arg^{77}-C3a. Human C3a (0.3 mg/ml) was converted to des-Arg^{77}-C3a by carboxypeptidase B (Boehringer Mannheim) in phosphate-buffered saline (pH 7.2) for 30 min at 37°C with a carboxypeptidase B/C3a ratio of 1:10 (wt/wt). Inactivation of carboxypeptidase B was performed with 50 mM ε-amino-*n*-caproic acid. In such conditions, 100% of the carboxyl-terminal arginine residue was removed from C3a, which was entirely converted into des-Arg^{77}-C3a as analyzed by V. R. Villanueva (21) by the use of an aminoanalyzer with fluorometric detection. Quantification was done by the method of the internal standard with carbamylputrescine.

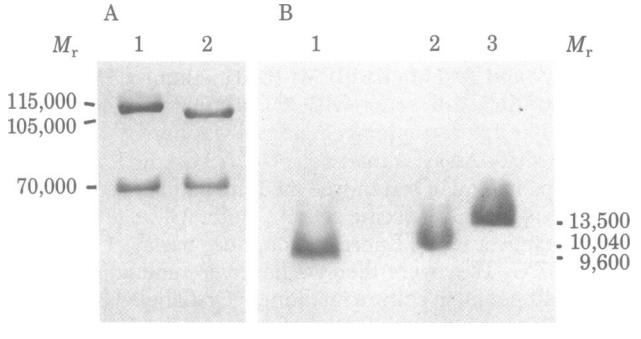

FIG. 1. (*A*) Analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis of highly purified human C3 (lane 1) and C3b (lane 2) in the presence of 2.5% 2-mercaptoethanol. Samples were run on a 10% polyacrylamide gel. (*B*) C3a fragment (lane 1) generated from C3 and separated from C3b was run on an 18% polyacrylamide gel in the presence of 2.5% 2-mercaptoethanol. Bovine neurophysine II (M_r 10,040) (lane 2) and cytochrome *c* (M_r 13,500) (lane 3) were used as markers, which spread also in this high polyacrylamide concentration.

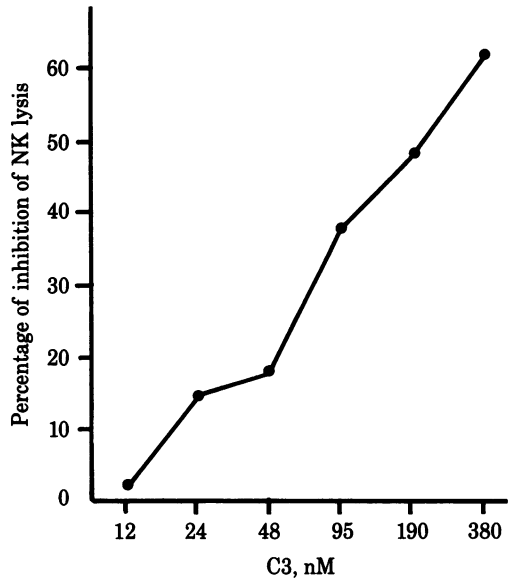

FIG. 2. Inhibition of mouse NK activity as a function of C3 concentration. Purified human-C3 (50 μl) was directly added into the 200-μl cell mixtures of C57BL/6 *nude* spleen cells and ^{51}Cr-labeled YAC-1 target cells. E/T ratio was 70:1.

Protein concentration was determined by the method of Bradford (22) with serum albumin as the protein standard.

RESULTS

Effect of Highly Purified Human C3 on *in Vitro* Mouse and Human NK Activity. Addition of C3 to cultures of mouse spleen cells and YAC-1 target cells during the cytotoxicity assay resulted in a dose-dependent inhibition of NK activity (Fig. 2). In this experiment, C3 at 180 nM (35 μg/ml) and 380 nM (70 μg/ml) induced a 50% and 63% inhibition of NK activity, respectively. Data from a total of 13 experiments, with four different preparations of purified C3 at 380 nM, have shown an inhibition of NK activity ranging from 42% to 100% with a mean

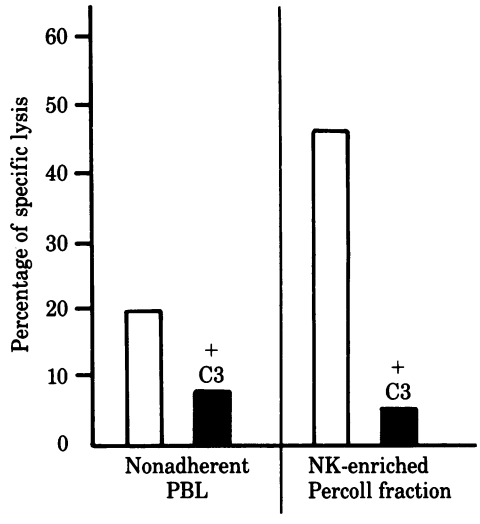

FIG. 3. Inhibition of human NK activity by C3. Human C3 (380 nM) was added directly to the mixtures of effector cells and ^{51}Cr-labeled K 562 target cells. Nonadherent peripheral blood lymphocytes were recovered after filtration through a nylon wool column and adherence to plastic Petri dishes. E/T ratio was 100:1. PBL, peripheral blood lymphocytes.

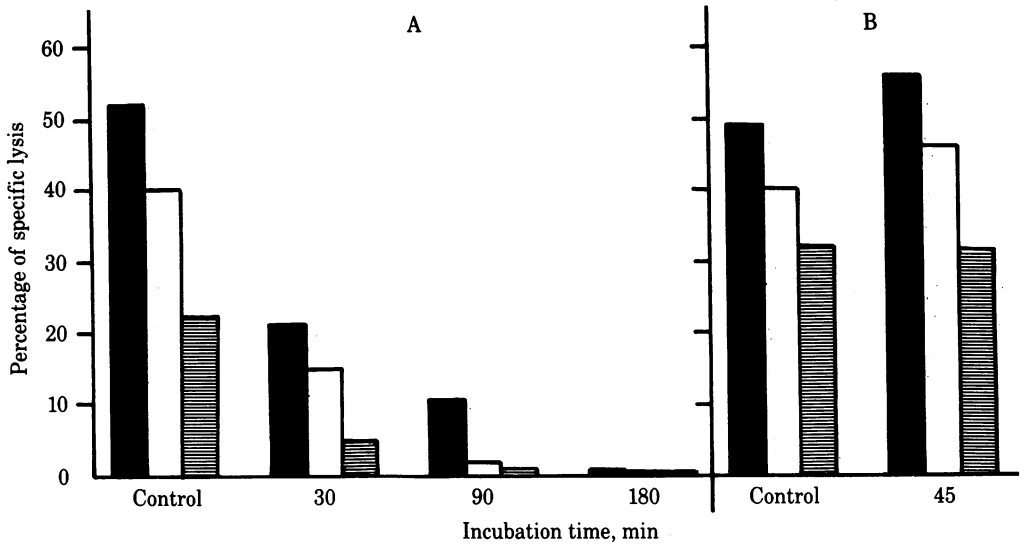

FIG. 4. Effect on NK activity of pretreatment of mouse effector or target cells with C3. (*A*) *Nude* spleen cells (5×10^6) in 0.6 ml were incubated for various periods of time with 150 nM C3, washed three times, and then tested in the NK assay. Control consisted of mouse spleen cells incubated for 180 min in control phosphate-buffered saline buffer. (*B*) ^{51}Cr-Labeled YAC-1 cells were incubated for 45 min with the same concentration of C3, washed three times, and then used in the NK assay. Control consisted of YAC-1 cells incubated for 45 min in phosphate-buffered saline. The percentage of spontaneous ^{51}Cr release was 12.2% from untreated cells and 14.7% from C3-treated cells. E/T: (■), 70:1; (□), 35:1; (▤), 17.5:1.

value of 63.5 ± 5.5% (SD). Similar results were obtained with NK cells from the LGL fraction of human peripheral blood lymphocytes fractionated on discontinuous Percoll density gradient (Fig. 3). In all experiments, cell viability was not affected by C3 and control buffer preparations, nor was observed cell agglutination, which could account for NK cell inhibition.

To test whether the presence of C3 was necessary throughout the whole cytotoxicity assay in order to mediate this effect, or whether prior interaction between cells and C3 was sufficient, the following experiments were set up. *Nude* mouse spleen cells (enriched for NK activity by running through nylon wool columns) were incubated for various periods of time with 190 nM C3, washed extensively, and then assayed for NK cytotoxicity. Results (Fig. 4*A*) show that a 30-min pretreatment of the effector cells with C3 led to 58% inhibition of NK activity, this inhibition being almost complete after 180 min of preincubation. In contrast, a 45-min preincubation of YAC-1 target cells with C3 neither decreased their susceptibility to NK lysis (Fig. 4*B*) nor modified their spontaneous ^{51}Cr release. These data indicate that C3-induced inhibition of NK activity is a time-dependent process resulting from the interaction with the effector-cell population and not with the target cells.

Table 1. Effect of C3 cleavage on mouse NK activity

Spleen cell treatment	Percentage of specific lysis ± SD	
	Exp. I	Exp. II
None	65.5 ± 5.8	49.3 ± 6.9
C3	48.2 ± 5.1*	37.1 ± 5.3†
C3 preincubation with trypsin		
3 min	34.6 ± 5.6*	19.6 ± 2.9*
30 min	53.8 ± 0.7‡	45.9 ± 1.0§
Control buffer¶	64.0 ± 2.2§	49.9 ± 3.4§

The cleavage products of 380 nM of C3 were directly added in the NK test. E/T was 100:1.
*$P < 0.01$.
†$P < 0.02$.
‡$P < 0.05$.
§Not significant.
¶Trypsin with soybean trypsin inhibitor.

Effect of C3 Cleavage Products on NK Activity. To further investigate the structural relationship between the C3 molecule and its biological effect on NK cells, trypsin cleavage of C3 was undertaken. In a first step, C3 was submitted for either 3 min or 30 min to limited trypsin digestion terminated by soybean trypsin inhibitor, and the resulting C3a/C3b or C3c/C3d mixtures were tested in the NK assay. The C3a/C3b mixture was more effective in inhibiting NK activity than the same concentration of the original preparation (Table 1). In contrast, inhibition provided by the C3c/C3d mixture was either minimal or nonsignificant. In a second step, the C3a and C3b fragments obtained in 3-min trypsin treatment were isolated in the presence of 1.5 M NaCl (Fig. 1 *A* and *B*) dialyzed against phosphate-buffered saline and then tested separately. C3a at 1 μg/ml was as effective as the intact C3 component at 75 μg/ml in inhibiting

Table 2. Effect of separated C3b, C3a, and des-Arg^{77}-C3a fragments on NK activity

Effector cell treatment	Percentage of specific lysis ± SD	
	Human LGL	Murine spleen cells
None	42.6 ± 1.8	61.8 ± 1.3
Control buffer*	44.8 ± 2.4	59.6 ± 3.2
C3a and C3b dialysis buffer	44.0 ± 3.8	60.3 ± 5.9
Whole C3, 75 μg/ml	33.1 ± 0.9†	45.8 ± 3.5†
C3a, 1 μg/ml	26.3 ± 5.7†	43.3 ± 6.0†
C3b, 100 μg/ml	41.3 ± 3.0‡	56.4 ± 2.8‡
None	34.3 ± 2.1	69.6 ± 3.6
Control buffer§	30.0 ± 0.5	68.3 ± 2.7
C3a, 10 μg/ml	6.3 ± 2.1†	45.7 ± 4.8†
des-Arg^{77}-C3a, 10 μg/ml	5.5 ± 1.5†	43.2 ± 3.5†

Human LGL or nude mouse spleen cells (1×10^6) were respectively mixed with 1×10^4 K 562 or YAC-1 target cells in the presence of the C3 components.
*Trypsin with soybean trypsin inhibitor.
†$P < 0.01$.
‡Not significant.
§Carboxypeptidase B with ε-amino-*n*-caproic acid.

NK activity displayed by human LGL and by murine spleen cells as well (Table 2). In contrast, C3b at 100 μg/ml, obtained from the original active C3 preparation, was ineffective. It should be noted that during all steps of C3a and C3b separation, trypsin/soybean trypsin inhibitor-containing buffer was completely devoid of nonspecific inhibitory effect in the NK assay. Therefore, the C3a fragment appears as the sole fragment endowed with the inhibitory effect shown by the C3 molecule on NK activity.

Comparative Effects of C3a and des-Arg^{77}-C3a. It is well established that the biological function of C3a, as anaphylatoxin, is expressed when the carboxyl-terminal arginine residue is present on this molecule. Removal of this amino acid residue by carboxypeptidase B generates des-Arg^{77}-C3a, which is no more active (13). According to these data, purified C3a was treated by carboxypeptidase B (the release of the arginine residue being controlled by an aminoanalyzer), and des-Arg^{77}-C3a was tested in the NK assay. Both C3a and des-Arg^{77}-C3a were found to inhibit NK activity at similar levels (Table 2).

DISCUSSION

In this report, we show that highly purified human C3 exerts an inhibitory effect on *in vitro* mouse and human NK activities. This effect is dose and time dependent, an average 50% inhibition being obtained by adding 190 nM C3 (35 μg/ml) in the NK assay or by preincubating the effector cells with the same concentration of C3 for 30 min at 37°C. Such a preincubation of the target cells did not result in NK cell inhibition, indicating that C3 acts at the effector cell population level and not at the target cell level. C3-induced inhibition of cytotoxicity appears to be peculiar to the NK system because, in our hands, the same C3 preparations were ineffective in the *in vitro* assay for alloreactive cytotoxic lymphocytes (not shown).

Analysis of the structural relationship between the C3 molecule and its biological effect on NK activity showed that the suppressive effect is carried by the purified M_r 9,600 fragment of C3, the C3a, which can be generated in 3 min by trypsin treatment from the amino-terminal part of the α chain of native C3. Identity of the C3a fragment was established on the basis of its M_r (9,600), its heat resistance, and its alkaline properties tested by electrophoresis.

As little as 1 μg of C3a per ml was sufficient to mediate this effect, whereas purified C3b generated under the same conditions did not display inhibitory activity, even used at a 100-fold higher concentration. This represents an important internal control for the role of C3 and C3a. Likewise, complexes of C3c and C3d produced during a 30-min trypsin cleavage were inactive. During such a prolonged trypsin cleavage, C3a was digested in turn as assayed by gel electrophoresis (not shown), which is in agreement with other reports (19).

The fact that C3 and C3a were both inhibitory in the NK assay raises the question of whether releases of C3a from the rest of the C3 molecule is necessary to mediate this effect. In this regard two hypotheses have to be considered.

First, the active site on the C3a fragment involved in the inhibitory activity would be carried by the C3a part of the α chain of C3 and by the cleaved fragment as well. In this hypothesis, the active site on the C3a fragment would remain stable whatever the conformational changes induced during its separation from the α chain. Second, the active site would be carried only by the cleaved C3a fragment, which implies the presence of proteinases at the effector population level able to cleave C3 and to generate the C3a fragment. This is supported by the increased inhibition provided by the 3-min trypsin-pretreated C3 compared to the same concentration of uncleaved C3 (see Table 1). Also, some of us have shown that proteinases take place in the specific binding of C3 on the surface of human B lymphoblastoid cells (23) and that specific proteinases can cleave C3 and C3b molecules on human erythrocyte membrane (24). Such proteinases have been described on C3b receptor-positive human cells; however, according to the majority of experimental work (25), NK cells are devoid of receptors for C3b fragments.

It should be noted that our experiments were performed with either human LGL depleted of cells expressing C3 receptors (macrophages, B lymphocytes, and erythrocytes) or, in some experiments (not shown), with *nude* mice spleen cells depleted of B cells (by filtration through nylon wool columns) and macrophages (by *in vitro* injection of silica and filtration of the spleen cells through Sephadex G-10 columns). However, with the limit of the methods used to purify NK cells, we cannot rule out the presence in the NK assay of contaminating cells able to release proteinases or carrying C3a receptors at their surface. Interaction of C3a with such C3a receptor-bearing cells could induce the release of a suppressive factor, as for the C3a-induced release of vasoactive amines from mast cells, resulting in typical histamine-mediated reactions in the surrounding cells (13). From these data, investigations of the possible presence at the NK cell surface of C3a receptors and proteinases specific for C3 molecules are needed.

In our experiments, removal of the carboxyl-terminal arginine by carboxypeptidase B from C3a molecule did not impair its inhibitory effect on NK cells. In contrast, it is well known that the anaphylatoxic activity of C3a requires the presence of this carboxyl-terminal residue (13).

The *in vivo* relevance of our results has to be analyzed. It is tempting to assume that C3a may play a regulatory role in the *in vivo* homeostasis of NK activity despite the presence of carboxypeptidase B in the plasma. C3 cleavage can be promoted through the classical and alternative pathways. The latter can be activated by certain viruses, bacteria, yeasts, virus-infected cells, and transformed cells (10). It is conceivable that C3a generated during such interactions when they occur *in vivo* might locally suppress NK activity. Moreover, C3-secreting cells, like monocytes (9) and lymphocytes (26), might in certain conditions exert a suppressive control on NK activity. For instance, the cleavage of C3 secreted by macrophages could account for the NK cell inhibition induced by these macrophages when stimulated by bacteria and polyanions (6, 7). However, because C3a is highly susceptible to trypsin digestion, we cannot rule out an *in vivo* destruction of C3a by trypsin-like proteinases in the plasma or at the surface of certain cells (27).

While writing this manuscript, we became aware of reports that C3 and C3a molecules are able to interfere with the *in vitro* antibody response of rat and human spleen cells (28, 29). This emphasizes the possible important role of C3a in the regulation of several immunological cellular functions.

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