A further link between innate and adaptive immunity: C3 deposition on antigen-presenting cells enhances the proliferation of antigen-specific T cells

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Abstract
Murine cells of the B lymphoblastoid line A20 and concanavalin A-elicited peritoneal macrophages are shown to activate and fix C3 fragments covalently when incubated in fresh, autologous serum under conditions allowing the initiation of the alternative complement pathway. For the detection of cell-bound C3, cytofluorimetry was performed using FITC-labeled F(ab')2 fragments of anti-mouse C3. Cell-bound C3 fragments are not internalized or shed by the cells under culture conditions for at least two hours. When the antigen-presenting capacity of serum-treated cells was tested using various antigens and experimental systems, augmentation of the proliferation of antigen-specific T cells was found. This enhancing effect was particularly pronounced at suboptimal antigen doses. The elevation of T cell proliferation induced by C3-opsonized antigen-presenting cells (APC) could be abrogated by F(ab')2 fragments of goat anti-mouse C3, suggesting the involvement of C3 receptors expressed by T cells in the process. Using the 7G6 mAb recognizing murine CR1/CR2, the presence of these complement receptors on activated T cells is demonstrated by cytofluorimetry and immunoprecipitation, as well. These results point to the role of C3 bound to acceptor sites on APC in the facilitation of antigen presentation, providing a further link between innate and adaptive immunity.

Introduction
Recent findings reported by several authors (reviewed in 1) clearly demonstrate that various elements of the innate immune system play an important role in initiating and directing the acquired immune response. The role of the complement system, particularly that of the third component, C3, and receptors binding its activation fragments have been shown to influence specific immune responses by various mechanisms (2,3). C3b bound to the antigen was found to enhance the response of T cells (4); the attachment of C3b to tetanus toxin has been demonstrated to induce the redistribution of peptide–MHC complexes, resulting in higher efficiency of antigen presentation (5); iC3b/C3dg bound to immune complexes generated with natural antibodies and a primary antigen was found to promote antigen uptake and the expression of co-stimulatory molecules (6). Recently, C3d has been identified as a ‘molecular adjuvant’ by its capacity to augment primary and secondary antibody responses in vivo (7). In all these studies the effect of C3 fragments conjugated to the antigen was investigated. Our aim is to reveal the role of cell-bound complement fragments in the process of antigen presentation.

It has been known for a long time that Epstein–Barr-virus-transformed human B lymphocytes are able to activate the alternative pathway of the autologous complement system (8,9). Production of C3 by human monocyte-derived macrophages and U937 cells, and deposition of C3 fragments on the membrane of these cells have also been described earlier (10,11). Recently HIV-infected T and monocytic cell lines were reported to activate the complement cascade (12,13) and human monocyte-derived macrophages infected with HIV-1 were shown to fix produced C3 on their surface (14). It had also been shown that freshly isolated, normal human B cells bear C3 fragments fixed to the cell membrane (15,16). Deposition of C3 fragments on the surface of these cells does
not lead to lysis, due to the complement regulatory proteins present in the cell membrane of all nucleated cells (17). While CR2 (CD21) was found to be the predominant acceptor molecule on the surface of the Raji cells and human B lymphocytes (16,18), the covalent attachment of C3 fragments has been demonstrated on cells lacking CR2 as well, i.e., on monocyte-derived macrophages and U937 cells (10,11), and on the human myeloid cell line P39 (19), suggesting the existence of C3 acceptor molecules other than CR2.

Regarding the deposition of C3 onto cells of non-human origin, much less data are available. As a consequence of complement activation, covalent fixation of C3 fragments had been demonstrated in the case of guinea pig macrophages (20), the mouse mastocytoma cells P815 and some mouse cell lines, including the B lymphoblastoid cell line A20 (21,22).

In the present study we further characterize C3 deposition on the surface of mouse cells as a consequence of activation of the alternative pathway of complement. An additional functional role of acceptor-bound C3 is described, revealing a further link between innate and adaptive immunity. We demonstrate that C3 fragments fixed to antigen-presenting cells (APC) enhance the proliferation of antigen-specific T cells by interacting with C3 receptors expressed on activated T lymphocytes.

**Methods**

**Sera**

Serum collected from BALB/c mice was freshly aliquoted and kept at −70°C until use. To block the classical pathway, human serum prepared in triplicates, followed by washing with RPMI medium. Dilutions were prepared in RPMI medium.

**Cytokine detection**

Samples of cells (at the concentration of 2×10^7 cells/ml) were incubated with the relevant fluorochrome-labeled antibody and analyzed using a FACSscan (Becton Dickinson, Mountain View, CA) instrument. As a control, isotype-specific antibodies were used.

**Antigen-presentation assay**

For the presentation of OVA by peritoneal macrophages, serum-treated or untreated cells were distributed in 96-well TC plates (Costar, Corning-Costar Europe, The Netherlands), at a density of 5×10^4 cells/well. Various dilutions of the antigen were added to the samples prepared in triplicates, followed by the addition of antigen-specific T cells (2×10^5 cells/culture) isolated from the lymph nodes of OVA-injected animals by panning on anti-mouse IgM-coated Petri dishes. After culturing for 4 days in RPMI medium containing 10% FCS, [3H]thymidine-pulsed samples were harvested and measured. For the presentation of influenza virus, hemagglutinin-derived peptide P4 was added at various dilutions to 2×10^4 A20 cells in each sample and 1×10^4 peptide-specific T hybridoma cells were added to the cultures prepared in triplicates. Stimulation of T cells was assessed by measurement of IL-2 in culture supernatants, using the IL-2-dependent HT-2 cell line. Stimulation indexes (SI) are given in the figures.

**Immunoprecipitation and Western blotting**

Cells were surface biotinylated and lysed as described by Cole et al. (25). Samples were precleared by Protein G beads and then immunoprecipitated using the mAb 7G6, reacting with mouse CR1/CR2, described and kindly provided by Dr Kinoshita (26). Samples were run on SDS–PAGE followed by electrophoretic transfer to nitrocellulose sheets. Proteins on the blots were revealed by HRP-conjugated streptavidin and DAB, using H_2O_2.

**Results**

C3 binds covalently to the surface of mouse cells as a consequence of alternative pathway activation of autologous complement

It has been demonstrated earlier that several types of cells and cell lines of various origin are able to activate complement
and fix C3 fragments covalently (8–16,18–22). Here we demonstrate that mouse peritoneal macrophages and cells of the B lymphoblastoid line A20 can also activate the alternative pathway of autologous complement and fix C3 fragments on the cell membrane. Figure 1 shows that incubation of Con A-elicited peritoneal macrophages and A20 cells in 10% fresh autologous serum results in deposition of C3 fragments on the cell surface. Membrane-bound C3 was monitored by cytofluorimetry, using FITC-labeled goat F(ab')2 fragments of anti-mouse C3. To test whether the complement fragments are fixed to the surface of the cells covalently, MA-treated serum was used. As it is shown in Fig. 1, C3 deposition was almost completely abrogated by inactivation of the thiol ester of C3 with MA, proving that the complement protein interacts with the membrane of the cells covalently. Experiments carried out using Mg-EGTA serum demonstrate that the alternative pathway of complement is activated by the cells. (Figure 2 shows data obtained using A20 cells.)

Studying the kinetics of the reaction, we found that incubation of A20 cells with serum for 60 min resulted in maximal deposition of C3 fragments (Fig. 3). The percentage of C3-bearing cells did not change in the next 2 h (the cytofluorimetric profile was the same as shown after incubation of the cells for 120 min), proving that no degradation or internalization of the covalently fixed complement fragment occurs.

Enhancement of antigen-presentation by APC preincubated with fresh serum—the role of covalently bound C3

The involvement of complement in the process of antigen presentation has been demonstrated by several authors. Arvieux et al. had shown earlier that C3 fragments covalently

Fig. 1. Covalent attachment of C3 fragments to the cell membrane of A20 cells (a) and peritoneal macrophages (b). Mouse cells treated with 10% fresh, autologous serum (shaded background) or MA-treated serum (strong line) for 60 min were washed and incubated with the FITC-labeled F(ab')2 fragment of anti-C3. The control consisted of cells treated only with the labeled antibody fragment. The x- and y-axes correspond respectively to the fluorescence intensity and the number of cells. Data shown are representative of four experiments.

Fig. 2. Activation of the alternative complement pathway leads to C3 deposition. Cells of the B lymphoblastoid A20 line were incubated in fresh, autologous serum diluted 10-fold with RPMI medium only and with medium containing Mg-EGTA. After washing, the cells were incubated with FITC-labeled F(ab')2 fragment of anti-C3. The control consisted of cells treated only with the labeled antibody fragment. The x- and y-axes correspond respectively to the fluorescence intensity and the number of cells. Data shown are representative of three experiments.

Fig. 3. Kinetic analysis of C3 deposition on A20 cells. Cells were incubated with 10% fresh, autologous serum for the indicated period. After washing, cells were incubated with the FITC-labeled F(ab')2 fragment of anti-C3. The control consisted of cells treated only with the labeled antibody fragment. The x- and y-axes correspond respectively to the fluorescence intensity and the number of cells. Data shown are representative of three experiments.
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cross-linked to the antigen enhances the proliferation of antigen-specific T cells (4), while Boackle et al. found that CR2 augments antigen presentation in immune individuals (27). Our earlier experiments have also shown that macrophages of C3-deficient guinea pigs present antigens less efficiently than APC isolated from normal animals (20). Based on these data and our results shown in Figs 1 and 3, we assumed that C3 bound to the cell membrane might also influence the process of antigen presentation. To test this, A20 cells and peritoneal macrophages pretreated with serum were used as APC. Three antigen presentation systems were set up using these cells and different antigens: macrophages and A20 cells were used to present OVA to T cells obtained from the lymph node of immunized mice, while A20 cells were used to present P4 peptide derived from the hemagglutinin of the influenza virus to the T hybridoma IP12-7. The SI values shown in Fig. 4 demonstrate that preincubation of APC with fresh mouse serum results in the enhancement of T cell proliferation in each sample and, particularly in the case of A20 cells presenting the influenza hemagglutinin-derived peptide, the effect of serum treatment was more pronounced in the case lower antigen concentrations.

To study whether covalently fixed C3 fragments play any role in this process, the extent of antigen-induced T cell proliferation was compared in cultures where the presenting capacity of macrophages and A20 cells pretreated with MA-treated serum was compared to the activity of cells preincubated in fresh serum. As Fig. 5 shows, APC pretreated with inactivated serum do not augment the proliferation of T cells, in none of the cases, showing that the presence of covalently fixed C3 fragments on the surface of APC is necessary for the enhancement of T cell proliferation.

Serum treatment has no effect on cell surface molecules of APC involved in antigen presentation

Results shown in Figs 4 and 5 demonstrate that antigen-induced T cell proliferation is enhanced by APC bearing covalently fixed C3 fragments on their cell membrane. One of the possible mechanisms by which serum-induced augmentation might occur is the enhanced expression of molecules important in the activation of antigen-specific T cells as a consequence of C3 fixation. To test if serum treatment of APC has any influence on the appearance of co-stimulatory or accessory molecules involved in the process of antigen presentation, the availability of several cell membrane molecules was compared before and after serum treatment of A20 cells and peritoneal macrophages. Table 1 shows that neither MHC class II molecules nor several other important structures known to be involved in the interaction between APC and antigen-specific T cells (e.g. B7-2, ICAM-1, CR1, CR2, LFA-1 and FcγRII) are influenced by the incubation of the cells with fresh, autologous serum. Interestingly, in the case of the B lymphoblastoid cells A20, CR1/CR2 molecules were not influenced by C3 deposition, either.

Inhibition of the serum-induced enhancement of T cell proliferation by C3-specific antibodies

Our results shown in Figs 4 and 5 and Table 1 point to the role of APC-bound C3 in the enhancement of antigen-induced T cell proliferation. To address the question whether augmentation of T cell activation is indeed mediated by C3 fragments, the assay was carried out in the presence of F(ab′)2 fragments of C3-specific polyclonal antibodies. As it is shown in Fig. 6, in this case the extent of T cell proliferation was similar to that

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Fig. 4. Enhancement of the proliferation of antigen specific T cells by serum-treated APC. A20 cells (a and c) and Con A-elicited peritoneal macrophages (b) were used to present various concentrations of OVA (a and b) and the influenza hemagglutinin peptide P4 (c) to T cells isolated from lymph nodes of OVA-injected mice (a and b) and the IP12-7 hybridoma (c). SI of samples containing APC treated with 10% serum (dark columns) and control APC (light columns) are shown at various antigen concentrations. Data shown are representative of three experiments.
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Fig. 5. Incubation of APC with MA-treated serum does not enhance the proliferation of antigen-specific T cells. A20 cells (a) and Con A-elicited peritoneal macrophages (b) were used to present OVA at the concentration of 3.2 (a) and 16 (b) µg/ml to T cells isolated from the lymph nodes of OVA-injected mice. SI of samples containing non-treated APC, i.e. cells incubated in medium (light column), APC treated with 10% fresh serum (grey column) and APC treated with MA-treated serum (dark column) are shown. Data shown are representative of three experiments.

Table 1. The expression of surface molecules on APC after treatment with 10% fresh, autologous serum

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<tr>
<th>Cell membrane molecules</th>
<th>Medium</th>
<th>Fresh serum</th>
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<tbody>
<tr>
<td>MHC class II</td>
<td>572</td>
<td>580</td>
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<tr>
<td>FcγRII</td>
<td>351</td>
<td>345</td>
</tr>
<tr>
<td>B7-2</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>LFA-1</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>ICAM-1</td>
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<td>51</td>
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<tr>
<td>CR1</td>
<td>45</td>
<td>47</td>
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<tr>
<td>CR3</td>
<td>172</td>
<td>165</td>
</tr>
<tr>
<td>Δ mean fluorescence of A20 cells</td>
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<td></td>
</tr>
<tr>
<td>MHC class II</td>
<td>811</td>
<td>805</td>
</tr>
<tr>
<td>FcγRII</td>
<td>562</td>
<td>565</td>
</tr>
<tr>
<td>B7-2</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>LFA-1</td>
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<td>85</td>
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<td>CR1,2</td>
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<td>79</td>
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<tr>
<td>Ig</td>
<td>65</td>
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mAb (26). Activated mouse T lymphocytes, however, have not been tested yet. We have addressed this question by cytfluorimetry and immunoprecipitation using the mAb 7G6, which recognizes CR1 and CR2 as well. As shown in Fig. 7, both the T cells isolated from the lymph nodes of immunized mice and cells of the T hybridoma used in our experiments react with the CR1/CR2-specific mAb. The presence of CR1/CR2 was demonstrated by immunoprecipitation, as well. (Figure 8 shows results obtained with IP12-7 cells.) In this case the lysate of surface biotinylated hybridoma cells was immunoprecipitated with 7G6 antibody. As shown in lane 4 of Fig. 8, the mAb specifically reacts with CR2 of ~150 kDa and with CR1 of ~190 kDa derived from the T hybridoma, similarly to C3 receptors isolated from spleen cells (Fig. 8, lane 2). In the case of the T cells, however, an additional protein of ~195 kDa also appears, which is specifically immunoprecipitated by the mAb 7G6 (Fig. 8, lane 4). The analysis of this species is in progress in our laboratory.

Discussion

Complement component C3 and receptors reacting with various activation fragments of this protein have long been implicated in immune responses (30). It had been shown that not only T cell-independent, but also T cell-dependent responses are strongly influenced by C3 and its receptors, particularly in conditions when the antigen is present in suboptimal doses (3). Moreover, increasing amounts of data reveal the role of various elements of innate immunity (including the complement system, as well) in directing and influencing adaptive immune responses (1).

Our data presented here provide an additional link between these two systems, pointing to the role of C3 in the enhancement of the adaptive immune response, particularly at suboptimal antigen doses. We demonstrate that as a consequence of activation the alternative pathway of the complement system, C3 fragments are deposited on the surface of APC, which in turn facilitate the contact with
activated T cells, resulting in the enhanced proliferation of antigen-specific lymphocytes. The covalent interaction of C3 with various cells (in the absence of membrane-bound antibodies) has been described by several authors; human B lymphocytes, B lymphoblasts, HIV-infected T cells, macrophages and the U937 line were found to activate and fix C3 (10,11,14–18). In the case of human B cells it had been demonstrated that CR2 is involved in the initiation of the alternative pathway activation and it had also been demonstrated that this receptor is the main acceptor molecule reacting with activated C3 (16,18). However, in the case of macrophages (which fix C3, but do not express CR2) the cell membrane molecule interacting covalently with the complement protein is still not identified. In our present experiments using mouse peritoneal macrophages it is clearly demonstrated that these cells can activate autologous serum via the alternative pathway and fix C3 fragments covalently, similarly

Fig. 6. Effect of anti-C3 F(ab')2 on serum-induced enhancement of T cell proliferation. A20 cells (a) and Con A-elicited peritoneal macrophages (b) were used to present OVA at the concentration of 16 (a) and 3 (b) µg/ml to T cells isolated from the lymph nodes of OVA-injected mice. SI of samples containing non-treated APC, i.e. cells incubated in medium, and serum-treated APC (light column), and those of cultured in the presence anti-C3 F(ab')2 (dark column) are shown. Data shown are representative of three experiments.

Fig. 7. Expression of CR1/CR2 on activated T cells. T lymphocytes isolated from the lymph node of OVA-injected mice (a) and cells of the IP12-7 T cell hybridoma (b) were labeled with FITC-conjugated mAb 7G6 recognizing CR1/CR2. The x- and y-axes correspond respectively to the fluorescence intensity and the number of cells. Data shown are representative of three experiments.

Fig. 8. Immunoprecipitation of CR1/CR2 from the extract of T hybridoma cells. Surface-biotinylated spleen cells (lanes 1 and 2) and cells of the IP12-7 hybridoma (lanes 3 and 4) were lysed in 1% NP-40 followed by immunoprecipitation using the mAb 7G6 bound to Protein G beads (lanes 2 and 4). Control samples (lanes 1 and 3) were prepared using Protein G beads only. Samples were run on 7.5% SDS–PAGE and the Western blot was developed after incubation with streptavidin–HRP conjugate, using DAB.
to A20 cells of B lymphoblastoid origin (Fig. 1). In an attempt to identify possible acceptor molecules on the surface of these cells, the presence of several molecules was tested (including CR1/CR2, MHC class II and co-stimulatory molecules) before and after serum treatment. The availability of these structures with the antibodies used, however, was not influenced by serum treatment (Table 1), suggesting that the C3 deposition involves other cell surface structures. These data also point to the possibility, that in the case of murine B cells CR2 does not serve as C3 acceptor, in contrast to its human counterpart.

Deposited C3 is present on the cell surface under culture conditions for hours, consequently it may influence several functions of these cells. We have investigated whether the very first step of the adaptive response might be influenced by these opsonized cells, i.e. the antigen-presenting capacity of macrophages and the B cell line A20 was studied. Our data demonstrate that C3 fixed to the surface of these cells causes the enhancement of the proliferation of antigen-specific T lymphocytes (Fig. 4). It has been proven that for the enhancement to occur, the covalent attachment of C3 is necessary, since in experiments, where MA-treated serum was used, the enhancement of antigen presentation could not be detected (Fig. 5).

Based on our data we suggest that APC-bearing acceptor-bound C3 are able to facilitate the contact with activated T lymphocytes by interacting with C3 receptors expressed on these cells as a consequence of stimulation. The exact biological role of C3 receptors on T lymphocytes has not yet been clarified. Earlier results obtained using murine cells show that, while C3 alone does not influence the proliferation of T cells, the complement protein (particularly when aggregated) significantly enhances the IL-2-dependent growth of these cells (31). We assume that in addition to the possible involvement of lymphokines in the process described above, the ‘bridge-forming’ capacity of C3 (32) might also play a role, as it had been demonstrated earlier in the case of various cytotoxic reactions (33–35). C3 receptors on mouse T cells have not been characterized yet. Now using the anti-CR1/CR2 mAb 7G6 we show the expression of these receptors on T cells isolated from the lymph node of OVA-injected mice and on the T hybridoma IP12-7 (Figs 7 and 8). By immunoprecipitation with the same antibody, CR1 and CR2 molecules of identical mobility to those found on spleen cells were identified in the extract of the T hybridoma. In addition to these C3 receptors, a protein of ~195 kDa is also specifically immunoprecipitated by the 7G6 antibody, suggesting that it is antigenically related to CR1/CR2. Further characterization and identification of the possible function of this molecule is in progress at our laboratories. The role of CR1/CR2 on T cells in the enhancement of antigen presentation is suggested by the finding that antibodies to C3 (blocking the interaction with the receptor) inhibit the proliferation of the antigen-specific lymphocytes (Fig. 6).

The phenomenon described in this paper is in line with the alarming role of the elements of innate immunity; the adaptive response is initiated/enhanced only if there is danger, e.g. a pathogen invades the organism, causing complement activation directly or via the activation of APC engulfing them. This activation may result in the deposition of C3 fragments on the surface of these cells and, in turn, acceptor-bound C3 fragments can interact with C3 receptor-bearing cells. Since activated macrophages are able to produce and fix C3 as well (10,11), these ‘self-opsonized’ APC may augment cell-to-cell contact at sites of immune reactions, e.g. in germinal centers (36). The synthesis of complement in various tissues and the local functions of complement (37) may underlie both protective and injurious roles.

Acknowledgements

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Abbreviations

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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>MA</td>
<td>methylamine</td>
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<td>OVA</td>
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References


