Amplification of the Antibody Response by C3b Complexed to Antigen Through an Ester Link

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Complement C3 has been described as playing an important role in the cell-mediated immune response. C3b has the capacity to covalently bind Ag and then to stimulate in vitro Ag presentation to T lymphocytes. To verify this observation in vivo, we prepared and purified covalent human C3b-Ag complexes using lysozyme (HEL) as Ag. The characterization of these HEL-C3b complexes indicates that they are representative of those susceptible to be generated in physiological conditions. Mice were immunized with 0.1 to 0.6 μg of either free HEL, HEL + C3b, HEL-C3b, or HEL + CFA. Response was assessed after two i.p. injections by quantification of specific Ab production. Immunization with either HEL-C3b complexes or HEL + CFA leads to anti-HEL IgG production whereas free HEL or HEL + C3b was ineffective. Either HEL-C3b or HEL + CFA immunizations led to a similar Ig subclass patterns, including IgG1, IgG2a, IgA, and IgM.

Our experiments provide the first evidence for modulation of specific Ab response by C3b when it is bound to Ag through a physiological-like link. Taken together with previous data concerning Ab response following recombinant HEL-C3d immunization, cellular events such as processing of C3b-Ag by APC and recognition by T lymphocytes, this present result underlines the importance of C3b and its fragments in stimulation of the immune system, through the multiplicity and complementarity of its interactions. *The Journal of Immunology, 1999, 162: 3647–3652.*

Evidence for close connections between innate and acquired immunity arose several years ago. The role of complement in Ab production in vivo was first observed using C3-depleted mice or guinea pigs with inherited or acquired C3 deficiency; such animals showed diminished Ab response to T cell-dependent Ag (1–4). The role of complement receptor (CR)3 in this impaired humoral immune response was pointed out by studies involving CD35 (CR1)- and CD21 (CR2)-deficient mice. These animals have a profound defect in T-dependent Ab response (5–7). Along the same line, also, administering anti-CD21 Ab (8, 9) or soluble recombinant CD21 to normal mice (10) can suppress their in vivo immune response.

The mechanisms leading to this suppressive effect remain unclear, but appear to be related to the characteristics of this protein, upon limited proteolysis, undergoes a conformational change leading to the rupture of an internal thioester and acquires the capacity for covalent binding to other proteins such as Ag. Such C3b-Ag complexes are able then to interact with C3b receptors present on APCs. We have previously studied the effects of binding C3b to TT on the toxin presentation; C3b-TT enhances TT internalization (11) and presentation (12) to TT-specific T lymphocytes (reviewed in Ref. 13). These studies suggest that, after proteolytic activation in areas of inflammation, C3 may play a role in delivering Ag to APCs. Other mechanisms may take place, which may involve CR-like CD21, known to interact on the cell surface with molecules involved in signal transduction such as CD19 (14–16) and CD81 (17, 18). B cell activation was found to be enhanced upon cross-linking of Ag receptor and CD19 (19), which could occur through C3b-Ag complexes, involving the CD21/CD19/CD81 complex. Moreover, CR are present on different APC, including dendritic cells, which are involved in the development of B-memory cells (20). Thus, C3b bound to Ag may lead to more efficient B cell priming, giving rise to enhanced humoral immune response.

A first approach to the study of the in vivo role of C3 used a recombinant lysozyme (HEL)-marine C3d fusion protein (21); in this case, C3d was shown to act as a molecular adjuvant. To extend this observation to C3b under conditions closest from natural, we developed an in vitro method to obtain covalent complexes between HEL and C3b through the active nuleophile group, after disruption of the intramolecular thioester of C3. In immune complexes, it has been found that C3b molecules bound to Ag by a covalent link (22), indicating that this represents physiological complexes. The C3b-Ag complexes, generated in vitro using such a covalent link and thus representing physiological-like complexes corresponding to those naturally occurring, were used to immunize mice. Analysis of the induced primary and secondary responses confirms the importance of C3 as a biologic immune modulator and its potential use as a tool for targeted modulation of the immune response.

Materials and Methods

Materials

Outdated human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble, France). Trypsin (t-rosylphenylalanylethromethane (TPCK)-treated), soya-bean inhibitor, 3,3′, 5,5′-tetrathemylbenzidine (TMB), and paranitrophenyl phosphate were from Sigma (St.
HEL-specific IgG1 (F10, ascitic fluid) were prepared in our laboratory. No mouse IgM was from Southern Biotechnology Associates (Birmingham, France). Peroxidase-conjugated rabbit anti-mouse IgG and goat anti-mouse IgA were from Immunotech (Marseille, France). Mouse IgA, IgM, and horseradish peroxidase-conjugated rat anti-mouse IgG and horseradish peroxidase-conjugated rat anti-mouse IgG1 were from Sigma. Alkaline phosphatase-conjugated rat anti-mouse IgG2a and horseradish peroxidase-conjugated goat anti-mouse IgM was from Southern Biotechnology Associates (Birmingham, AL). Rabbit HEL-specific IgG, mouse IgG2a (asctic fluid), and mouse HEL-specific IgG1 (F10, ascitic fluid) were prepared in our laboratory. No cross-reaction between the different Ig and anti-Ig was observed.

**HEL-C3b complex formation**

C3 and C3b were purified according to Refs. 23 and 24, respectively. The concentration of the purified proteins was estimated from absorbance at 280 nm, using E1%, 1 cm = 9.7 (25). HEL-C3b complexes were obtained as previously described for C3b-TT (24), with the following modifications. Three milligrams of purified C3 (1 mg/ml in 20 mM KH₂PO₄, 0.15 mM NaCl, 20 mM EDTA, pH 7.2) was precipitated in the presence of 13% polyethylene glycol (w/v, final concentration). After 30 min of stirring at 4°C, the mixture was centrifuged at 4°C for 12 min at 12,700 g, and the pellet was resuspended in 250 μl of 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.2 containing 15 mg of HEL (HEL:C3 ratio = 5:1, w/w). Cleavage by trypsin (enzyme:C3 ratio = 1:500, w/w) and addition of soya-bean trypsin inhibitor (enzyme:inhibitor molar ratio = 1:2) were as in Ref. 24. The proteins were diluted with 1 ml PBS containing 0.5 M NaCl and centrifuged at 4°C for 12 min at 12,700 × g to remove insoluble material.

**HEL-C3b complex purification**

To eliminate free HEL from the HEL-C3b complexes, the mixture obtained above was applied to a Sephadex G75 column (18 × 960) (Pharmacia, Saint Quentin, France) equilibrated in PBS, at a flow rate of 3 ml/h. Fractions of 1.3 ml were collected, and their absorbance at 280 nm was monitored. The fractions of a first peak corresponding to HEL-C3b complexes were pooled (15–18 ml) and applied to a column (10 × 270) of Phenyl Sepharose Fast Flow (Pharmacia) equilibrated in PBS, at a flow rate of 6 ml/h. The column was washed with the same buffer, and proteins were eluted using a 0–50% (w/w) linear gradient of glycerol in PBS (total volume 72 ml), followed by 50 ml of 50% glycerol in PBS, to assure complete elution. Fractions (1.2 ml) were collected in siliconed tubes and analyzed on SDS-PAGE. Free C3b is eluted first. Fractions containing HEL-C3b were pooled and concentrated by ultrafiltration on PM 10 (Amicon, Epernon, France). The concentration of C3b complexed to HEL was estimated after analysis on SDS-PAGE in reducing conditions; quantification of α’ chain of C3b complexed to HEL, using purified C3b as standard, was conducted by Fluorimag (Molecular Dynamics, Bondoufle, France).

**SDS-PAGE analysis**

Samples for electrophoresis were prepared in reducing conditions and analyzed on 7.5% polyacrylamide slab gels as described (26). After electrophoresis, proteins were stained using AgNO₃ (27) or Sypro Orange according to the manufacturer’s indications.

**Western blots**

Electrophoretic transfer of proteins from SDS-PAGE to PVDF membrane was conducted in 0.1 M CAPS buffer (pH 11), containing 10% methanol. For immunoblotting, PVDF membranes were incubated with stirring, 15 h at 4°C in 5% dry milk in PBS, pH 7.2 (PBS). Incubation with Abs and washing were done in PBS with 0.1% Tween-20. Detection was performed using peroxidase-conjugated goat anti-rabbit Abs with the enhanced chemiluminescence system (ECL, Amersham). Western blots were revealed using Hyperfilm-ECL (Amersham).

**Treatment with hydroxylamine**

Proteins were incubated with an equal volume of 2 M hydroxylamine in 0.1 M Tris-HCl buffer (pH 9.6) for 3.5 h at 37°C.

**Injections in mice**

BALB/c mice (7 wk old) were purchased from IFFA-CREDO (L’Arbresle, France). Groups of five mice were injected (i.p.) with 400 μl of PBS alone or containing HEL (0.6, 0.3, or 0.1 μg) in different combinations: HEL in CFA (HEL + CFA, 1/1v in stable emulsion), HEL and free C3b (HEL + C3b), or HEL covalently linked to C3b (HEL-C3b) (Fig. 1A). In the two latter cases, the C3b amounts injected are the same, taking into account the presence of free C3b in HEL-C3b complexes (HEL:C3b molar ratio = 1:1.4). The mice were injected (i.p.) on day 0, boosted (i.p.) on day 28, and bled on days 11 and 40.

**Ab assays**

HEL- and C3-specific Ig were assayed by either direct or indirect ELISA. Coating, washing, and incubations were as described (28). For direct ELISA, microtiter Luxlon plates (CML, Nemours, France) were coated with C3 (1 μg/ml) or HEL (10 μg/ml). For indirect ELISA, microtiter plates (DYNATEC, St. Cloud, France) were coated with polyclonal rabbit HEL-specific IgG (100 μg/ml). After washing the plates, HEL (2 μg in 200 μl PBS-Tween 0.1%) was added and incubated 2 h at 37°C.

The different Ig classes and subclasses were revealed using either horse-radish peroxidase- or alkaline phosphatase-conjugated Abs, using TMB or paranitrophenyl phosphate, respectively, as substrate. The data are expressed as the mean of relative units (RU) corresponding either to the dilution giving 50% of the maximal value obtained with the standard curve, which is established with pooled sera from hyperimmunized mice, or, in the case of weak response, to the OD obtained for a 1/500 serum dilution.

**Statistical analysis**

Statistical analysis was done using the Pearson’s test with StatView statistical software (Abacus Concept, Berkeley, CA). The statistical significance level was defined as < 0.05.

**Results**

**Characterization of HEL-C3b complexes**

HEL-C3b complexes, obtained as described in Materials and Methods, were analyzed on SDS-PAGE (Fig. 1A). Under reducing conditions, three major protein bands were observed. Two of them correspond to reduced C3b (α’, M₉ = 110,000; β’, M₆ = 75,000). The M₃ of the third band (120,000) is consistent with a covalent association between the α’ chain of C3b and one molecule of HEL (M₃ = 14,400). To confirm the composition of this band, a Western blot was performed using anti-C3 and anti-HEL (Fig. 1B). The presence of both C3 and HEL in the protein band at 120,000 allowed us to conclude that it corresponds to a covalent association between C3b and HEL (HEL-C3b complexes). To define the binding of C3b to HEL, we incubated HEL-C3b complexes with 1 M hydroxylamine (Fig. 1C). This treatment resulted in total hydrolysis of the complexes, which demonstrates an ester linkage between C3b and HEL. Free HEL is not present in the complexes, as demonstrated by the lack of low M₃ after staining or after Western blot using anti-HEL Abs. Relative evaluation, after staining, of the two protein bands at 110,000 and 120,000 (α’ and α’-HEL, respectively) indicates that about 70% of C3b was incorporated into the complexes whereas 30% was free.

The use of a standard curve, obtained using purified C3b, allowed us to quantify the complexes. About 0.5% of the C3 initially used to form HEL-C3b complexes was recovered in the purified complexes.

**Binding of C3b to HEL leads to enhanced anti-HEL Ab formation**

Groups of five mice received two injections of HEL in different combinations. The relative levels of anti-HEL Ab elicited in each mouse were quantified by ELISA. No IgM anti-HEL primary response was detected in any of the immunized mice (data not shown). A weak but significant IgG primary response was observed for two mice injected with the higher dose of HEL + CFA; a 1/500 serum dilution led to OD of 0.26 and 0.27 whereas the same dilution of serum from hyperimmunized mice led to an OD equal to 2.1. These were the animals that produced the highest levels of anti-HEL IgG during the secondary response. At all Ag doses tested (0.6 to 0.1 μg), mice that received HEL + CFA or...
HEL-C3b complexes developed an anti-HEL IgG secondary response (Fig. 2). There is, however, a variability of response among HEL-C3b-immunized mice; in each group, even with the highest dose, some individuals did not respond, suggesting that this is not due to limited quantity of Ag. It should be noted that, in HEL-responding mice, the Ab level observed is a function of the Ag dose injected, this effect being similar whether the Ag is HEL-C3b or HEL

C3b alone or bound to HEL elicits efficient anti-C3 Ab formation

In our experiments, mice receiving HEL + C3b did not produce anti-HEL IgGs, showing that C3b must be directly bound to HEL to induce an anti-HEL response. C3b itself, being human, may be considered as an Ag itself and could elicit an Ab response. Therefore, we measured the level of anti-C3b IgG elicited in each mouse having received injections of C3b together with free HEL (HEL + C3b) or complexed to HEL (HEL-C3b) (Fig. 3). In four of five cases, an Ab response against C3b was obtained. This clearly demonstrates that at least four of five mice receiving C3b + HEL, that do not produce anti-HEL IgG, have indeed received the Ag mixture and were immunocompetent.

Ig classes and subclasses elicited during the secondary response

To compare the Ig secretion pattern in mice injected either with HEL-C3b or HEL + CFA, we analyzed, in addition to IgG, IgM and IgA levels. IgM was never detected. A weak IgA response was observed in some cases (Table I). Among mice injected with HEL-C3b, only 5 of 15 presented an IgA response whereas, when animals received HEL + CFA, a greater number (11 of 14) produced a detectable level of IgA. IgG subclasses were also investigated. In every case, IgG1 levels were high, reflecting the preeminence of this subclass (Fig. 4), whereas a weak IgG2a response was observed (Table I). Four of fifteen and 13 of 14 mice presented an IgG2a response after injection of HEL-C3b and HEL + CFA, respectively. In both cases (injection of HEL + CFA or HEL-C3b), there was a correlation between IgG and IgG1 levels ($p < 0.0001$, correlation coefficients $= 0.810$ and 0.898, respectively) whereas no correlation was observed between IgG and IgG2a ($p = 0.178$ and 0.103, correlation coefficients $= 0.278$ and 0.284, respectively).
Discussion

Several lines of evidence support a role for C3b in cell-mediated immune response. In vivo experiments are now required to establish whether C3b covalently linked to Ag is sufficient for induction of an optimal immune response to low Ag concentration and, from that, to deduce the mechanisms involved. First, we produced and purified covalent Ag-C3b complexes. We used HEL, a well-known and widely used nontoxic molecule, as Ag. Analysis of the complexes obtained (m.w., immunoblot, effect of nucleophilic agent) were consistent with an ester linkage between one molecule of C3b (α-chain) and one molecule of HEL. The absence of free HEL in our preparations allowed us to use HEL-C3b complexes as Ag. Low amounts of free C3b are mainly due to the weak difference in molecular mass and in the hydrophobicity property between C3b and HEL-C3b. The free C3b has no significant effect in our system as demonstrated by the immunization with HEL + C3b.

Such covalent complexes were used to analyze in vivo the role of C3. Dempsey et al. (21) observed an adjuvant effect when two or three copies of C3d were associated to Ag in a recombinant HEL-C3d fusion protein. Immunization with HEL-C3b covalently complexed by an ester link allowed us to approximate more closely physiological conditions.

We used a heterologous system as HEL complexed to human C3b was injected to mice. We chose human C3b since it is available in larger amounts than its murine counterpart. An interaction between human C3b and murine CR on B and T lymphocytes has been demonstrated (29–32). Moreover, internalization of HEL-C3b by murine B cells can be partially inhibited by human C3b-C3b dimers whose internalization is inhibited specifically by polyclonal anti-human C3b Abs (data not shown). These observations strongly argue in favor of human C3b-murine CR interactions.

Table I. Secondary response anti-HEL IgA and IgG2a titers elicited in immunized mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>HEL-C3b</th>
<th>HEL + CFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 µg</td>
<td>0.3 µg</td>
</tr>
<tr>
<td>IgA</td>
<td>1  0.06  0  0</td>
<td>0.22  0  0</td>
</tr>
<tr>
<td></td>
<td>4  0.25  0  0</td>
<td>0.22  0  0</td>
</tr>
<tr>
<td>IgG2a</td>
<td>1  0  0  0.03</td>
<td>0  0.11  0.05</td>
</tr>
<tr>
<td></td>
<td>4  0.08  0  0</td>
<td>0.05  0.35  0.09</td>
</tr>
</tbody>
</table>

* Sera from mice immunized with HEL (0.6 µg, 0.3 µg, or 0.1 µg) in CFA (HEL + CFA) or complexed to C3b (HEL-C3b) were tested for anti-HEL IgA and IgG2a. Serum titers are expressed as the OD obtained by ELISA reaction using a 500-fold dilution of the serum. Positive control using direct coat with IgA:OD = 2.1 and IgG2a:OD = 2.3.

** The relative error in these numbers is always <10%.

*** ND, not done because of the death of the mouse.

Figure 4. Anti-HEL IgG and IgG1 subclass titers elicited in mice (secondary response). Sera from mice having received HEL (0.6–0.3–0.1 µg) in CFA (HEL + CFA) or complexed to C3b (HEL-C3b) were tested for total anti-HEL IgG and anti-HEL IgG1 subclasses. Total IgG and IgG1: serum titers are expressed as RU (see Materials and Methods). The relative error is always less than 10%.
The absence of a primary response against HEL in our experiments was probably due to the low dose of Ag injected. Preliminary experiments allowed us to detect a weak primary response in mice injected with 10 μg of HEL + CFA (data not shown). The magnitude of the secondary immune response to HEL-C3b complexes was much greater than that elicited by free HEL. An important IgG-anti-HEL response was observed using only 0.1 μg HEL bound to C3b whereas, in its free form, 20 μg of HEL were required to induce detectable IgG anti-HEL secretion (data not shown).

C3b binding to CR or to anti-C3b membrane Ig does not potentiate per se an immune response against HEL. In fact, the adjuvant effect is obtained only when C3b is covalently bound to Ag. A good anti-C3b response was induced despite the low amounts of C3b injected (1.8–5.5–11 μg). This observation underlines the dual behavior of human C3b in our system, acting simultaneously as an Ag and a CR ligand. As shown in Fig. 3, one mouse does not produce HEL-specific Abs when injected with HEL-C3b. One explanation for this nonresponsive mouse could be a defective administration of the Ag; this is supported by the fact that this mouse displays the lowest anti-C3 level.

In our experiments, anti-HEL response was mainly provided by IgG, whatever the Ag used (HEL-C3b or HEL + CFA). IgM and IgA are not, or weakly, detectable. It was previously shown that different helper T lymphocyte subpopulations (Th1 and Th2) are activated, depending partly on the nature of the Ag (33) and adjuvant (34) used. Thus, we evaluated the specific IgG1 and IgG2a subclasses elicited by HEL-C3b and HEL + CFA. In both cases, mice developed a predominantly Th2-like response with a strong IgG1 production. IgA and IgG2a production was partially enhanced when HEL was emulsified in CFA.

Our data provide the first evidence for the adjuvant effect in vivo of C3b when directly linked to Ag through the typical thioester activation mechanism. This effect could be mediated by different nonexclusive mechanisms that could be independent or dependent of CR. Firstly, C3 may interfere with Ag processing. This is sustained by the observation of a delay in Ag proteolysis when it is associated to C3b (12) and an inhibition by C3c of Ag proteolysis by lysosomal proteases (35). Moreover, this C3b-Ag binding leads to an increase in SDS-stable HLA-DR class II molecules, which can occur independently of the Ag uptake in CR-negative cells (36). Secondly, CR expressed on most APC (B cells, dentritic cells, macrophages; Ref. 37) enhance the Ag uptake, leading to increased presentation (11). Third, C3 fragments-CR interaction induce an increase of Ig synthesis by B cells, as demonstrated in vitro (38). Of CR, CD21 seems to be the main protagonist because its ability to associate with CD19. The CD19/CD21 complex is involved in signal transduction and regulation of the humoral immune response by B cells (14, 16, 18). C3b-Ag binding to CD21/CD19 complex could modify B cell activation. This effect could be enhanced by simultaneous interactions between Ag and Ag receptor, on the one hand, and C3b and CR, on the other, during the secondary response, leading to cross-linking of the Ag receptor and CD21/CD19 complex (11).

In the system described by Dempsey et al. (21), the authors used recombinant HEL-C3d fusion proteins as Ag. Though using a homologous system (Ag fused with murine C3d before injection in mice), HEL needed to be linked with two or three copies of C3d to enhance the immune response. Therefore, this effect could be due to coligation of several CD21 (as mentioned in Ref. 39). Moreover, the use of C3d instead of C3b limits C3-CR interactions to CD21 whereas C3b and its derivatives can interact with CD35, CD11b, CD11c, as well as CD21. Though the role of CD21 seems to predominate in the interaction between C3b-Ag and APC (11, 40), cooperation between CD35 and CD21 is highly likely and could influence the C3b-Ag uptake, processing, and signal transduction. A complex between CD35 and CD21, excluding CD19, has been described on human B cells (15, 18) and may be coupled to signal transduction pathways different from CD21/CD19. Another important factor for the immune response elicited by C3b-Ag is the nature of the link between the two molecules. Likely, the ester bond used in our study leads to physiological treatment of the C3b-Ag complexes, undoubtedly different from the peptide bond in the HEL-C3d recombinant protein.

A similar adjuvant effect was suggested for α2M, which belongs to the same family as C3b, C4, and FcPZ since these proteins possess an internal thioester bond (41). An enhancement of immune response was observed in rabbits immunized with α2M-Ag complexes (42) that could be due to α2M receptors present on cells involved in the immune response (43). Thus, the adjuvant effects of C3b and α2M could result from similar mechanisms.

Since most B cells carry CR, CD35 may facilitate Ag localization within germinal centers and influence the generation of B memory cells. A C3-dependent stimulation of unprimed B cells has already been observed (44), and this point is under investigation in our system.

Our results, obtained in vivo with C3b-Ag complexes, conclusively demonstrate the importance of C3 in stimulation of the immune response. This stimulation is dependent on an intricate set of interactions between immunocompetent cells and C3b-Ag complexes. Analysis, at all levels, of the mechanisms of such interactions are now required to gain knowledge of the role of C3, and this may lead to the development of more effective tools for immune response optimization.

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References


