Improvement of long-lasting response and antibody affinity by the complexation of antigen with complement C3b

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Abstract

Complement protein C3 plays a major role in cell regulation and immune response. This last point is mainly due to C3’s capacity to act as a bifunctional link between antigen and immunocompetent cells. In a previous work, we have reported the adjuvant effect produced by linking C3 fragments to antigen. In this paper, we characterize the long-term secondary antibody response induced by C3b–antigen complexes. Mice were immunized using either a protein (hen egg lysozyme) or an ovalbumin-derived peptide as antigen. We compared the secondary response elicited by these antigens covalently linked to C3b or emulsified in complete Freund’s adjuvant (CFA). Our results provide evidence that C3b linkage induces better long-term adjuvant effect than CFA, resulting in the production of a higher specific IgG titer with a better affinity.

Introduction

Complement protein C3 plays a major role in the complement activation pathway and in the immune response [reviewed in (1,2)]. Increasing evidence from in vitro as well as in vivo experiments demonstrates the involvement of C3b and its fragments in various mechanisms leading to antigen recognition and antibody response. Thus, several intracellular events are influenced by C3b covalent binding to antigen, including antigen uptake (3), intracellular traffic (4), proteolysis (5) and binding of antigenic peptides to MHC class II molecules (6). In vivo studies using animals with inherited or acquired C3 deficiencies as well as mice lacking CD21/35 (Cr2±/± mice) [reviewed in (1)] underline the importance of C3 in the development of the antibody response to either T-dependent or -independent antigens. The impaired immune response observed in such animals could be related to a defect in (i) B cell maturation (7), (ii) antigen localization and generation of germinal centers (GC) in lymphoid organs (7), and/or (iii) interaction between follicular dendritic cells and B cells (1).

More recently, experiments using C3b covalently linked to hen egg lysozyme (HEL) in 1:1 complexes (C3b–HEL) as antigen demonstrated the adjuvant role of C3b and/or its fragments (8). Similar results were obtained with C3d fragments either in a C3d–HEL recombinant protein (9) or in a C3d–sHA DNA vaccine (10). Whereas one C3b molecule coupled to antigen had an adjuvant effect, several C3d molecules were needed to enhance the immune response.

The quality of an immune response is characterized by different parameters including the concentration and persistence of antibodies, as well as their affinity. Until now, no data are available about the long-lasting immune response induced by C3b–antigen complexes. Furthermore, studies using Cr2±/± mice are contradictory, as either an increase (11) or a decrease (7) in antibody affinity in the absence of C3 receptors was reported. In this paper, we use protein or peptide as antigen, covalently linked to C3b through an ester bond (8). These complexes, which resemble those naturally occurring in vivo following complement activation, were injected into BALB/c mice; persistence and affinity of the resulting specific IgG antibodies were compared with those elicited following injection of antigen emulsified in complete Freund’s adjuvant (CFA).

Methods

Reagents, proteins and buffers

Turkey egg lysozyme (TEL), 3,3’,5,5’-tetramethylbenzidine, BSA (fraction V), chicken egg ovalbumin (OVA) and horse-radish peroxidase-conjugated rabbit anti-mouse IgG were...
from Sigma (St Louis, MO). Tween 20 and HEL were from Merck (Laboratoire Merck-Clevenot, Paris, France). Peptide OVA273–288 was purchased from Synt:em (Nõmes, France). NH₄SCN was from Prolabo (Paris, France). Human C3 and C3b were purified as described (12). HEL–C3b complexes were obtained as previously described (8). In these complexes, C3b and HEL are associated through an ester link, with 1:1 molar stoichiometry. Peptide–C3b complexes were obtained using Sulfo-MBS (Pierce, Rockford, MD) according to the manufacturer’s instructions. Mouse HEL-specific mAb D44-1, D1-3, D11-15 and F10 (13) were kindly provided by C. Leclerc (Institut Pasteur, France). PBS was 139 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄ and 8.2 mM Na₂HPO₄, pH 7.2. NH₄SCN was in 0.1 M phosphate buffer, pH 6.0.

**Immunizations**

BALB/c mice (7 weeks old) were purchased from IFFA-CREDO (l’Arbresle, France). Groups of three to five mice were injected i.p. on day 0 with 0.3 μg HEL or 110 ng OVA273–288 either in CFA [HEL + CFA or peptide + CFA, 1:1 (v/v) in stable emulsion] or covalently linked to C3b (HEL–C3b or peptide–C3b) in 300 μl final volume. Mice were boosted on day 28 under the same conditions. They were then bled regularly from day 35 to 420. Serum was obtained after clotting at room temperature and centrifugation for 5 min at 10,000 g. Hyperimmune serum was obtained from mice injected with

20 μg HEL emulsified in CFA using the protocol described above, 45 days after immunization.

**Antibody assays**

HEL- and OVA-specific IgG were assayed by ELISA, as described in (8). Plates were coated with HEL (10 μg/ml) or OVA (20 μg/ml) respectively. Absorbance at 450 nm was determined using a Titertek Multiskan (Labsystems, Eragny/Oise, France). Anti-HEL IgG titers are expressed as relative units (RU) corresponding to the dilution giving 50% of the maximal absorbance. This maximum was obtained from the standard curve established using pooled sera from HEL-hyperimmunized mice. Due to the low level of specific IgG titers in the case of peptide immunization, data from ELISA tests were expressed as absorbance value at 450 nm of a 1/250 dilution of the serum. For competition assays, sera were preincubated for 15 min at room temperature in the presence of 0.6 μg/ml of HEL or TEL before ELISA.

**Avidity determination**

Relative antibody affinity/avidity was determined using the chaotropic thiocyanate ion in ELISA assays as described (14). Affinity index represents the molar concentration of thiocyanate required to reduce the initial optical density by 50%.

**Statistical analysis**

Statistical analysis was done using the t-test with StatView (version 5.0) statistical software. The statistical significance level was defined as P < 0.05.

**Results**

**HEL–C3b promotes long-term antibody production**

We previously showed that, after a maximum around day 45, specific IgG titers elicited by two injections of HEL emulsified in CFA (HEL + CFA) decreased rapidly, whereas two HEL–C3b injections led to a continuous increase of anti-HEL response. Thus, around day 160, we observed similar IgG titers in the case of peptide immunization, data from ELISA tests were expressed as absorbance value at 450 nm of a 1/250 dilution of the serum. For competition assays, sera were preincubated for 15 min at room temperature in the presence of 0.6 μg/ml of HEL or TEL before ELISA.

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Thus, although the specific IgG level is weaker when the antigen is a peptide compared to a protein, in both cases covalent binding of C3b induces a better long-term response than emulsion in CFA.

**HEL–C3b improves anti-HEL avidity**

An important component of the immune response is the quality of the antibody elicited. To compare the affinity/avidity of anti HEL IgG elicited either by HEL + CFA or HEL–C3b, we used the chaotropic thiocyanate ion in ELISA, as previously described (14). To validate this method of affinity/avidity measurement, we determined in our system the relative avidity of a panel of monoclonal anti-HEL antibodies with known binding constants (13). These mAb include D44-1 (K_b = 1.4 x 10^7 M), D1.3 (K_b = 27 x 10^7 M), D11-15 (K_b = 31 x 10^7 M) and F10 (K_b > 10 x 10^7 M). As shown in Table 1, a good correlation was observed between the affinity index determined in our assays and the known K_b of each tested mAb. The affinity index of anti-HEL IgG from hyperimmune serum is similar to those obtained with the highest affinity mAb.

This method was used to measure the affinity/avidity of anti HEL IgG produced at different times (days 90, 154 and 420) after immunization with HEL emulsified in CFA or linked to C3b. The values obtained within each group did not significantly vary along the period tested (P > 0.05) (data not shown), suggesting that, in our case, there is no affinity improvement of specific IgG after day 90, whatever the adjuvant used (CFA or C3b). The values obtained at different times for each type of mice were therefore grouped. As shown in Table 2, when immunization is performed with HEL–C3b, the affinity index is 30% higher than when immunization is performed with HEL + CFA (1.322 versus 1.006, P = 0.009).

**Epitopes recognized after immunization by HEL + CFA or HEL–C3b are slightly different**

To determine if there was any difference in the epitopes recognized by the specific antibody elicited by HEL + CFA or HEL–C3b injection, we used a competition assay as described in Methods with either purified, native HEL or TEL as competitor. As shown in Fig. 2, soluble HEL and TEL are better competitors for IgG elicited by HEL–C3b than HEL + CFA. This result suggests that antigen recognition differs according to the nature of the immunogen: coupling HEL to C3b leads to a better recognition of the native antigen than CFA.

**Discussion**

Our data provide the first evidence that covalent binding of C3b to antigen induces a better long-term stimulation of the immune response than the classical adjuvant (CFA). Moreover, the specific antibody exhibits a better affinity when elicited by C3b–HEL compared with HEL + CFA. These two parameters (long-lasting response and affinity) are of major importance for the protection against pathogens. Long-term immune response depends on different parameters which may be influenced by C3b (and its fragments) when covalently linked to antigen: (i) the generation of memory B cells, (ii) their re-stimulation upon exposure to antigen and (iii) their differentiation into antibody-secreting plasma cells.

Earlier studies suggested that C3b is involved in the generation of memory cells (7,15), but a better induction of such B cells during the primary response by C3b compared to CFA does not seem to be accountable for the observed improvement of the secondary response: we have shown that priming with HEL + CFA followed by a boost with HEL–C3b results in a 3- to 4-fold higher anti-HEL IgG titer than priming with HEL–C3b followed by a boost with HEL + CFA or two injections of HEL + CFA (16). The enhancing effect of C3b on secondary response was also observed in another study (17). Finally, the antibody titer during the secondary response is not always correlated with the frequency of antigen-specific memory B cells present at the time of boosting (18).

An important feature in the secondary immune response is the retention of antigen on follicular dendritic cells in the GC.
allowing continuous antigenic stimulation of memory B cells and protection against apoptosis (19). Binding of C3b on antigen could enhance its persistence on the surface of follicular dendritic cells via complement receptors (CR), increasing the re-stimulation and the survival of memory B cells (7). In addition, it is now well established that C3b binding to CR could trigger other membrane proteins involved in B lymphocyte activation/function, such as CD19 through a CD19–CD21 complex [reviewed in (20)]. In this case, it has been shown that the full co-stimulatory function of the CD19–CD21 complex requires its cross-linking to the antigen receptor, as would occur in the presence of C3b–antigen. Moreover, in vitro experiments demonstrated that enhancement of B cell response by CD21–35-BCR co-ligation is selective for the co-ligating antigen (21).

Antibody-forming cells (AFC) are essential for maintaining long-term antibody response: as the half-life of secreted antibody is ~3 weeks (22), a sustained elevated antibody level depends on the continuous presence of plasma cells. These cells could be either short-lived and continuously generated from memory B cells (23) or long-lived (24). This last type of AFC secretes antibody for extended periods of time in the absence of memory B cells and antigen (25). Whereas short-lived AFC are mainly present at early times after immunization, long-lived AFC are observed during later stages of the humoral immune response (24). The mechanisms involved in the induction, differentiation and survival of AFC are poorly understood. CD21 could play an important role in the induction and differentiation of AFC as it is highly expressed on transitional B cells of type 2 as well as on their mature B cell descendants (26). Moreover, signaling through CD21/35 could regulate the number and longevity of antigen-specific plasma cells by modulating the activity of transcriptional factors and proteins involved in B cell differentiation and survival such as NF-κB, Flip (27) and Bcl-2 (19). Finally, the mechanisms which regulate the migration of AFC from lymph nodes to bone marrow (BM) are poorly understood and we cannot exclude a role of CR signaling in AFC homing.

In addition to an enhanced long-lasting IgG titer, C3b linking to antigen induces an increase in specific antibody affinity. This result is in agreement with another study using a DNA vaccine encoding a sHA–(C3d)3 fusion protein for immunization (28). Moreover, indirect evidence for the implication of C3b in affinity maturation was obtained by Wu et al. (28) using NP-HSA conjugate as antigen. In their conditions, a limited affinity maturation was elicited in Cr2±/± mice as compared to normal animals. Our results demonstrate the importance of C3b when linked to antigen in B cell maturation as it results in an increase in antibody affinity even when compared to CFA. How does it work?

First, C3b could be involved in antigen receptor diversification in mature B cells as V(D)J recombinase activity in splenic B lymphocytes is regulated by cell-surface receptor signaling (29); thus, co-ligation of the BCR with CR by C3b–antigen complexes could stimulate BCR editing. However, if specific V(D)J frequencies are modified within the pool of splenic B cells in Cr2± mice, BCR somatic hypermutations are intact in GC B cells from these mice (11), minimizing the potential role of C3b at this level.

Second, interaction of C3b–antigen complexes with CR on AFC could induce these cells to leave GC before they are fully differentiated and migrate to the BM where terminal differentiation occurred (30). Furthermore, increased numbers of BM AFC were correlated with persistent titers of serum antibody (30). Signaling through CR could enhance selective retention and survival of high-affinity AFC and/or antibody secretion by these cells.

It should be noted that experiments with Cr2±/± mice address only the role of CD21/35; other C3 fragments receptors are expressed in these animals, such as CD46 or CD55 on B lymphocytes [reviewed in (31)], and CD11c or CD11b on macrophages and dendritic cells (32), and can therefore influence the humoral response. The use of C3b–antigen complexes in our experiments allowed the potential interactions of C3b and C3b fragments with all their receptors.

Our experiments using antigen competition in ELISA raise the question of differences in epitope generation between antigen coupled to C3b and antigen in CFA. Antibodies elicited by C3b–HEL exhibit a better recognition of native HEL (soluble) than those obtained after HEL + CFA injections. This finding suggests that the form of antigen alters its conformation/processing and results in different repertoires of induced antibodies.

Together, these results argue that antigen linking to C3b improves the quality of a humoral immune response and thus have implications for vaccine design.

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Abbreviations
AFC antibody-forming cell
BM bone marrow
CR complement receptor
GC germinal center
HEL hen egg lysozyme
HEL-C3b C3b covalently linked to HEL
Kb equilibrium binding constant
OVA albumin from chicken egg
RU relative unit
TEL turkey egg lysozyme

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