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14. Dissociation rates were determined by incubating renatured RNA and CBP2 for 1 hour to ensure complete binding and subsequently trapping any protein that dissociated by addition of heparin to a final concentration of 20 to 500 μg/ml. Fraction RNA bound was analyzed by filter binding (*4*). Dissociation rates were independent of heparin concentration. Endpoints were obtained from reactions in which the heparin was added before CBP2.
15. We estimate the equilibrium dissociation constant for protein binding to the core state either (i) by correcting the observed $K_d$ for the fraction of properly folded RNA or (ii) from the elemental rate constants for the core⇄core$^{CBP2}$ interconversion: (i) The observed $K_d$ is 0.8 nM (*4*), whereas the equilibrium constant for formation of the core state is 0.07 (*3*). Thus, the $K_d$ for binding to the core is equal to (0.8 nM) × [0.07/(1+0.07)] = 50 pM. (ii) The reverse and forward rates for formation of the core$^{CBP2}$ state are taken as the rate of dissociation of the CBP2-Δ5′ domain RNA complex (Fig. 4) and the rate constant for formation of core$^{CBP2}$ at 40 mM $Mg^{2+}$, respectively; $K_d$ = (0.12 $min^{-1}$)/(3.5 × $10^9$ $M^{-1}$ $min^{-1}$) = 30 pM.
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26. (i) $k(2° \rightarrow core)$ = 0.3 $min^{-1}$ (Fig. 2). The rate constant for the reverse reaction for this step, $k(core \rightarrow 2°)$, was calculated from the rate constant for the forward reaction divided by the equilibrium constant (*3*), (0.3 $min^{-1}$)/(0.07) = 4 $min^{-1}$. (ii) $k(core \rightarrow core^{CBP2})$ = 3.5 × $10^9$ $M^{-1}$ $min^{-1}$ was determined at 40 mM $MgCl_2$ and provides an estimate for the value at 7 mM $MgCl_2$. The rate constant for the reverse reaction, $k(core^{CBP2} \rightarrow core)$, is taken to equal the dissociation rate for the Δ5′ domain–CBP2 complex (0.12 $min^{-1}$; Fig. 4). (iii) The equilibrium constant for the core$^{CBP2}$⇄E$^{CBP2}$ transition is given by the ratio of dissociation rate constants for the bI5- and Δ5′-domain–CBP2 complexes [W. W. Cleland, *Biochemistry* **14**, 3220 (1974); data from Fig. 4], (0.12 $min^{-1}$/0.009 $min^{-1}$) = 13. $k(core^{CBP2} \rightarrow E^{CBP2})$ must be faster than 2 $min^{-1}$, the rate of splicing of the E$^{CBP2}$ state (*4*).
27. We thank P. Bevilacqua for discussion. Supported by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research (K.M.W.) and by the Howard Hughes Medical Institute (T.R.C.).

24 July 1995; accepted 29 December 1995

# C3d of Complement as a Molecular Adjuvant: Bridging Innate and Acquired Immunity

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**An optimal immune response should differentiate between harmful and innocuous antigens. Primitive systems of innate immunity, such as the complement system, may play a role in this distinction. When activated, the C3 component of complement attaches to potential antigens on microorganisms. To determine whether this alters acquired immune recognition, mice were immunized with a recombinant model antigen, hen egg lysozyme (HEL), fused to murine C3d. HEL bearing two and three copies of C3d was 1000- and 10,000-fold more immunogenic, respectively, than HEL alone. Thus, C3d is a molecular adjuvant of innate immunity that profoundly influences an acquired immune response.**

The decision of the acquired immune system to respond to an antigen may be based not only on what is non-self, but also on what is infectious and of potential danger to the host (*1*). How the immune system makes this latter determination is not clear because the antigen receptors that are distributed among different lymphocyte clones generally cannot distinguish between noxious and innocuous antigens.

Systems of innate resistance to infection evolved before acquired immunity and are triggered by certain biochemical characteristics that are shared by microorganisms but not by higher forms of life. Complement is a plasma protein system of innate immunity that is activated by microorganisms in the absence of antibody (*2*). One consequence of activation is the covalent attachment of fragments of the third complement protein, C3, to the activator, and two of these fragments, C3dg and C3d, bind to CR2 (CD21) on B lymphocytes. CD21 may have B cell–stimulating functions because it associates with CD19, a B cell membrane protein that amplifies B cell activation (*3*) and is required for normal T cell–dependent, B cell responses (*4*). In support of this, depleting mice of C3 or blocking the binding of ligand to CD21 raises by approximately 10-fold the threshold dose of antigen required to elicit antibody (*5*, *6*). Therefore, the complement system may be an innate immune system that provides information to the acquired immune system in an attempt to classify antigens according to their potential hazard.

To determine whether the immunity-enhancing function of complement is mediated solely by the attachment of C3d to antigen and, if so, the magnitude of this effect of C3d, we prepared recombinant model antigens of hen egg lysozyme (HEL)

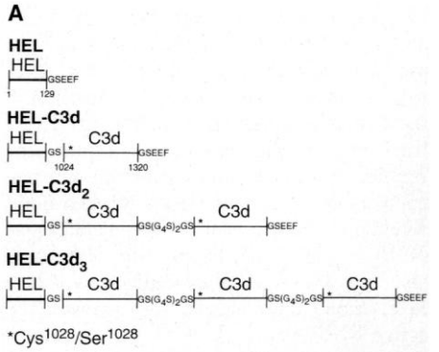


**Fig. 1.** Recombinant HEL and HEL-C3d fusion proteins. (**A**) Recombinant proteins comprising amino acids 1 to 129 of HEL alone and fused to one, two, or three copies of amino acids 1024 to 1320 of the C3d region of murine C3 were prepared (*7*). Amino acids in addition to those present in the native proteins are Gly (G), Ser (S), Glu (E), Phe (F), and Ser that was substituted for Cys (C) at position 1028 of C3 to avoid the presence of a free sulfhydryl in the recombinant proteins. (**B**) The recombinant proteins were purified from culture supernatants of transfected cells and assessed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining. Size markers are on the left in kilodaltons.

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alone and of HEL fused to one, two, or three copies of murine C3d (*7*) (Fig. 1). The recombinant proteins were bound with equivalent affinities by HyHEL-9 (*8*), a conformation-sensitive monoclonal antibody to HEL, and by HEL-specific B cells from transgenic mice (*9*) expressing an HEL antibody (anti-HEL) (HyHEL-10) that binds to a distinct epitope. Thus, fusion to C3d did not alter at least two epitopes of HEL. The ability of the C3d components of the fusion proteins to bind to CD21 was determined by competitive binding assays with Raji B lymphoblastoid cells expressing CD21 (*10*). In two experiments, 50% inhibition of specific binding of [$^{125}$I]HEL-C3d$_2$ occurred with 185 to 225 nM HEL-C3d, 8 to 20 nM HEL-C3d$_2$, and 1.5 to 2.2 nM HEL-C3d$_3$, respectively. The correlation between the avidity of the binding reaction and the number of C3ds suggests that each C3d in the fusion proteins was capable of interacting with CD21.

Coligating CD21 or CD19 to membrane immunoglobulin (mIg) with receptor-specific monoclonal antibodies lowers the threshold at which mIg stimulates an increase in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (*3*). B lymphocytes from mice expressing transgenes encoding mIgM and mIgD having a dissociation constant of 2 nM for HEL (*9*) were loaded with indo-1, incubated with incremental concentrations of the recombinant HEL proteins, and assayed for changes in $[Ca^{2+}]_i$ by flow cytometry (*11*) (Fig. 2). HEL caused a dose-related increase in $[Ca^{2+}]_i$, with the threshold being 7 nM. The presence of one, two, and three copies of C3d on HEL lowered the threshold to 0.7 nM, 0.07 nM, and 0.007 nM, respectively, which indicates that each C3d enhanced by 10-fold the ability of HEL to induce this cellular response. A saturating concentration of 7G6 antibody to CD21 (*12*) caused the threshold for the response of HEL-C3d$_2$ to revert to that of HEL. B cells from nontransgenic mice did not respond to HEL or HEL-C3d$_{1-3}$. The changes in $[Ca^{2+}]_i$ induced in nontransgenic B cells by antibody to mIgM were not altered by the presence of 7 nM HEL-C3d$_3$. Incubation of transgenic B cells with 3 nM HEL and HEL-C3d$_3$ for 10 min resulted in 51% and 47% saturation of the anti-HEL sites, respectively, as assessed by the subsequent binding of fluorescein-conjugated HEL. These results indicate that attachment of C3d to HEL did not improve binding to the transgenic B cells, presumably because of the high affinity of their antigen receptors for HEL. Therefore, the enhanced $[Ca^{2+}]_i$ responses reflected augmented signaling through C3d-dependent recruitment of the CD21-CD19 complex.

We compared the relative immunogenicity of HEL to that of HEL-C3d$_2$ and HEL-C3d$_3$ by immunizing mice with incremental amounts of the recombinant proteins subcutaneously in phosphate-buffered saline (PBS) (*13*). In preliminary experiments, HEL-C3d$_1$ was found to suppress inconsistently the primary IgG1 anti-HEL response, and the basis for this effect is unclear. The highest dose of HEL, 500 pmol, was the threshold at which unmodified antigen induced an IgG1 response after primary immunization (Fig. 3). In contrast, only 500 fmol of HEL-C3d$_2$ and 50 fmol of HEL-C3d$_3$ were required to induce comparable initial IgG1 responses. These doses of the three recombinant proteins also were the threshold amounts necessary for inducing immunologic memory, as indicated by the accelerated and augmented IgG1 responses occurring after primed mice were challenged with 50 pmol of HEL in incomplete Freund's adjuvant (IFA). Furthermore, the generation of immunologic memory to HEL by all recombinant proteins indicates that these responses are dependent on T cells.

The immunity-enhancing function of C3d was compared to that of complete Freund's adjuvant (CFA) (*13*). The threshold doses for eliciting initial IgG1 responses were 50 pmol for HEL in CFA and 500 fmol for HEL-C3d$_3$ in PBS, respectively (Fig. 4). The amounts required for inducing memory were 5 pmol and 50 fmol for HEL in CFA and HEL-C3d$_3$ in PBS, respectively. Therefore, attaching three molecules of C3d to the antigen is 100-fold more effective in lowering the threshold for acquired immune recognition than the action of the potent adjuvant, CFA.

The participation of CD21 in the IgG1 response to HEL-C3d$_3$ was determined. Mice were administered intraperitoneally 300 μg of 7G6 anti-CD21 or an isotype-matched control antibody 24 hours before immunization with 500 fmol of HEL-C3d$_3$ in PBS subcutaneously. The IgG1 anti-HEL titer at day 29 was less than 25 relative units (RU) for the 7G6-treated mice and 4610 RU for the control mice. Analysis on day 29 by flow cytometry of splenic lymphocytes stained with 7G6 anti-CD21 and 1D3 anti-CD19 (*14*), respectively, indicated that treatment with 7G6 had eliminated detect-

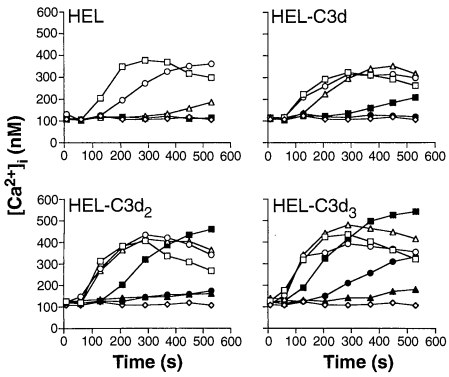


**Fig. 2.** Stimulation by recombinant HEL and HEL-C3d$_{1-3}$ of increases in $[Ca^{2+}]_i$ in HEL-specific B cells. Splenic lymphocytes from transgenic mice with B cells expressing mIgM and mIgD specific for HEL were loaded with indo-1 and incubated with incremental concentrations of HEL, HEL-C3d, HEL-C3d$_2$, and HEL-C3d$_3$, respectively: 70 nM (□), 7 nM (○), 0.7 nM (△), 70 pM (■), 7 pM (●), 0.7 pM (▲), and buffer alone (◇). Changes in $[Ca^{2+}]_i$ in B cells were monitored by flow cytometry (*11*).

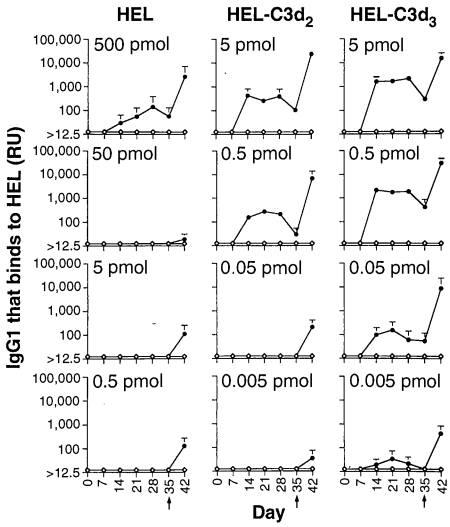


**Fig. 3.** The IgG1 anti-HEL response in mice immunized with recombinant HEL antigens subcutaneously in PBS. On day 0, groups of four mice were administered buffer alone (open symbols) or incremental amounts of HEL, HEL-C3d$_2$, or HEL-C3d$_3$ (closed symbols) subcutaneously in PBS. The mice were boosted on day 35 with 50 pmol of HEL intraperitoneally in IFA, as indicated by the arrow. Sera were assayed by ELISA for HEL-specific IgG1, and titers are expressed as relative units (RU) (*13*).

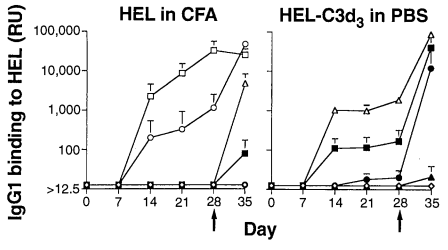


**Fig. 4.** Comparison of the effects of CFA and C3d on the immunogenicity of HEL. On day 0, groups of five mice were administered 500 pmol (□), 50 pmol (○), 5 pmol (△), 500 fmol (■), or 50 fmol (●) of HEL or buffer alone (◇) subcutaneously in CFA. Other groups of five mice were administered 5 pmol (△), 500 fmol (■), 50 fmol (●), or 5 fmol (▲) of HEL-C3d$_3$ or buffer alone (◇) subcutaneously in PBS. All mice were boosted on day 28 with 50 pmol of HEL in IFA as indicated by the arrow. The serum titers of HEL-specific IgG1 are expressed as RU (*13*).

able CD21 on B cells without diminishing their expression of CD19.

Mice were co-immunized subcutaneously in PBS with 25 μg of keyhole limpet hemocyanin (KLH) alone or in the presence of 0.5 pmol of HEL-C3$d_3$. The IgG1 response to KLH on day 14 was not altered by HEL-C3$d_3$, which indicates that C3d modifies the response only to the antigen to which it is bound.

These findings demonstrate that the immunity-augmenting function of the complement system is mediated solely by a discrete molecular modification of antigen, attachment of C3d, and that the magnitude of enhancement may be as great as 10,000-fold, which is far greater than even that of CFA. Three mechanisms may mediate this function. First, the association of CD21 with CD19, which is tyrosine-phosphorylated when mIg is ligated and binds phosphatidylinositol-3 kinase (*15*) and Vav (*16*), links the complement receptor to cytosolic effectors of signaling that amplify activation of B cells. The important role of CD19 in the humoral immune response recently has been exemplified in mice lacking this B cell protein (*4*). Second, the avidity with which low-affinity, unprimed B cells bind antigen is probably enhanced by the interaction of CD21 with C3d. Third, CD21 is expressed on follicular dendritic cells that may promote the development and maintenance of memory B cells (*17*).

The complement system, through the adjuvant effect of C3d, may select antigens for recognition by the acquired immune system. This function would be complementary to that of other components of innate immunity, such as macrophages, natural killer cells, and mast cells, which produce cytokines that may determine the character of an acquired immune response by directing the development of T helper type 1 and type 2 cells. Together, these humoral and cellular elements enable the acquired immune system to respond appropriately to potentially noxious foreign antigens. Their manipulation may lead to more effective strategies for vaccination.

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9. The anti-HEL mIgM-IgD transgenic mice MD4 (*18*) were maintained on a C57BL/6 background. The $F_1$ mice were produced by crossing MD4 with CBA/Ca (Harlan, Bicester, UK). Animal experiments were done according to procedures regulated under the Project License issued by the U.K. Home Office.
10. Raji cells, $3 \times 10^7$ to $4 \times 10^7$ cells/ml, were incubated for 45 min at 4°C with 1 nM [$^{125}$I]HEL-C3$d_2$ (specific activity, $9.1 \times 10^5$ cpm/μg) and incremental concentrations of the HEL-C3d fusion proteins, 500 nM $(CR2)_2$-IgG1 (*6*), or IgG1 in PBS containing 0.1% bovine serum albumin. The cells were centrifuged through a dibutyl-diiso-octyl pthalate cushion, and the amount of $^{125}$I bound to the cell pellet was determined.
11. Transgenic and nontransgenic splenocytes, $6 \times 10^6$ cells/ml in 10% fetal calf serum + RPMI + 50 μM 2-mercaptoethanol, were loaded with 2 μM indo 1-AM, washed, and stained with 10 μg/ml of phycoerythrin (PE)-conjugated anti-CD43 (Pharmingen). Changes in $[Ca^{2+}]_i$ were monitored by flow cytometric analysis (FACStar, Becton Dickinson) of PE-negative cells after addition of incremental concentrations of recombinant proteins.
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13. All mice were 4- to 8-week-old CBA/Ca males purchased from Harlan. Recombinant antigens were diluted in PBS containing 0.1% globulin-free murine albumin (Sigma) and administered in this buffer alone or after emulsification with equal volumes of IFA or CFA. Primary immunizations of 0.1 ml were administered subcutaneously to the right rear flank. Secondary immunizations of 0.1 ml in IFA were administered intraperitoneally. HEL-specific IgG1 was assayed by enzyme-linked immunosorbent assay (ELISA) (*18*) using horseradish peroxidase–conjugated goat antibody to mouse IgG1 (Southern Biotechnology Associates) and microtiter plates coated with HEL (Sigma). Use of recombinant HEL and HEL-C3$d_{1\text{-}3}$ as antigens in the ELISA gave comparable results. The data are represented as mean relative units (RU) + 1 SD based on a standard curve established with pooled sera from hyperimmunized mice.
14. 1D3 is a rat monoclonal IgG2a antibody to mouse CD19 (I. Krop *et al.*, *Eur. J. Immunol.*, in press). For fluorescence-activated cell sorting analysis, splenocytes were stained with 5 μg/ml of 7G6, 1D3, or control antibody followed by PE-conjugated goat antibody to rat F(ab)$_2$ (CalTag).
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19. We thank G. Fey for the murine C3 complementary DNA, K. Drickamer for the dog pre-pro-insulin signal sequence, R. Smith for help in producing recombinant proteins, A. Venkitaraman for the A71d vector, and C. Milstein for the YL 1/2 antibody. Supported by the Wellcome Trust (D.T.F. and P.W.D.), NIH grant AI07247 (P.W.D.), and the Medical Research Council (M.E.D.A.), Howard Hughes Medical Institute (S.A. and C.C.G.), and NIH grant AI19512 (C.C.G.).

14 September 1995; accepted 23 November 1995


# *DPC4*, A Candidate Tumor Suppressor Gene at Human Chromosome 18q21.1


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About 90 percent of human pancreatic carcinomas show allelic loss at chromosome 18q. To identify candidate tumor suppressor genes on 18q, a panel of pancreatic carcinomas were analyzed for convergent sites of homozygous deletion. Twenty-five of 84 tumors had homozygous deletions at 18q21.1, a site that excludes *DCC* (a candidate suppressor gene for colorectal cancer) and includes *DPC4*, a gene similar in sequence to a *Drosophila melanogaster* gene (*Mad*) implicated in a transforming growth factor–β (TGF-β)–like signaling pathway. Potentially inactivating mutations in *DPC4* were identified in six of 27 pancreatic carcinomas that did not have homozygous deletions at 18q21.1. These results identify *DPC4* as a candidate tumor suppressor gene whose inactivation may play a role in pancreatic and possibly other human cancers.


The development of human cancer involves the clonal evolution of cell populations that gain competitive advantage over other cells through the alteration of at least two distinct classes of genes: proto-oncogenes and tumor suppressor genes (*1*). Tumor suppressor genes are characterized by alterations that inactivate both alleles (*2*). Many tumor suppressor genes are inactivated by intragenic mutations in one allele accompanied by the loss of a chromosomal region containing the other allele, termed loss of heterozygosity (LOH). Mapping of homozygous deletions within regions showing a high frequency of LOH has been a critical step in the discovery of several tumor suppressor genes (*3*).

Human pancreatic ductal carcinomas