Enhancement of T cell-independent immune responses *in vivo* by CD40 antibodies

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In this report we describe a potentially powerful method for vaccinating infants against encapsulated bacterial pathogens such as *Haemophilus influenzae, Streptococcus pneumoniae* and *Neisseria meningitidis*. High levels of antibody directed against the polysaccharides of the bacterial capsule are normally protective[1–3]. Unfortunately, the capsular polysaccharides are T cell-independent antigens (TI); lacking T-cell help, they induce only weak, predominantly IgM antibody responses, with infants responding especially poorly[4]. T-cell help, given to B cells during responses to protein antigens, causes stronger antibody responses and isotype switching to the IgG isotypes. T-cell help is mainly mediated through ligation of the B-cell surface antigen, CD40, by its cognate T-cell ligand, CD154 (ref. 5–9). Here we show that administering anti-CD40 monoclonal antibody to mice, along with pneumococcal polysaccharide, provides a substitute for T-cell help and results in the generation of strong, isotype-switched antibody responses, which are protective. The work points the way toward a possible effective and inexpensive means of protecting susceptible groups against important bacterial pathogens.

Immune responses to capsular polysaccharide from *S. pneumoniae* were studied using a murine model. Intraperitoneal immunization of BALB/c mice with type 3 pneumococcal capsular polysaccharide (PS3) alone induced weak IgM and IgG3 responses against the antigen (Fig. 1*a*). This is typical of the response to TI type II antigens in mice (humans produce IgM and IgG2). Administration of the anti-CD40 antibodies 1C10 or 4F11 with PS3 induced small but significant rises in specific IgM and IgG3, whereas, remarkably, 1C10 induced significant polysaccharide-specific IgG1, IgG2a and IgG2b responses. These isotypes are not normally seen in response to TI type II antigens. The 1C10 antibody would appear to have successfully mimicked T-cell help by inducing high antibody titers and isotype switching *in vivo*. The anti-polysaccharide response was extremely persistent, with antibody being detected at high titers 14 weeks after the single immunization (Fig. 1*a*). No memory response against the polysaccharide was induced, as a second injection of polysaccharide alone failed to boost antibody responses (data not shown).

Streptococcus pneumoniae has more than 80 different capsular polysaccharide types, and any vaccination would be expected to induce protective immunity against a number of the more common serotypes[3]. A current pneumococcal vaccine, Pneumovax II (Merck, Sharp and Dohme), consists of 23 different polysaccharides. Mice were immunized with this 23-valent vaccine and 1C10. Inclusion of the CD40 antibody successfully generated strong IgG responses against randomly chosen polysaccharide types 4, 8, 12 and 19 (Fig. 2). Such isotype-switched responses were also generated against the two other antigens we examined, types 3 and 14, and the pattern of IgG isotype distribution against the polysaccharide was similar to that shown in Fig. 1*a* (data not shown). Therefore, 1C10 enhances responses to TI type II antigens other than PS3.

Given that administration of CD40 antibody mixed with polysaccharide would not restrict or even target CD40 ligation to antigen-specific B cells, we anticipated polyclonal activation of B cells with a resultant rise in total serum immunoglobulin levels. Indeed, 1C10 and PS3 induced some splenomegaly and 2- to 4-fold rises in total serum immunoglobulin levels (Fig. 1*b*). This, however, should be contrasted with up to 50-fold rises in specific antibody levels, indicating that polysaccharide-specific antibody production was preferentially enhanced. This skewing toward specific antibody is also not unexpected as it reflects *in vitro* findings. *In vitro*, although 1C10 could induce B-cell proliferation in the absence of stimulation through the antigen receptor, proliferation was synergistically enhanced by such costimulation[10]. The 4F11 antibody, which largely lacks agonist activity *in vitro*, did not enhance responses as efficiently as 1C10, demonstrating an association between adjuvant activity *in vivo* and B-cell activation *in vitro*.

Although induction of polyclonal antibody responses is not in itself a major problem, these responses may increase the risk of autoantibody production. Serum samples taken from mice treated with 500 μg of 1C10 14 days or 14 weeks earlier were screened against Hep-2 cells for autoantibodies. No autoantibodies were detected, except in the positive control serum from MRL mice (data not shown). However, we cannot rule out the risk of autoantibody production. Any potential risk should be greatly reduced by lowering the dose of CD40 stimulator, and we have achieved some enhancement of specific immune responses against PS3 with doses as low as 50 μg 1C10. We would envisage that the dose could be reduced much further by efficient colocalization of antigen and stimulator, perhaps through coemulsification or coentrapment in microparticles or liposomes. Indeed, in separate work, administering a T cell-dependent antigen attached to 1C10 has allowed the stimulatory dose of antibody to be cut from 500 μg to below 1 μg, while maintaining marked enhancement of specific immune responses without polyclonal activation (manuscript in preparation).

During a T cell-dependent response, CD40 ligation is necessary for switching to IgG isotypes, but various cytokines also play important roles[8,9,11–13]. It was, therefore, intriguing that such isotype-switched responses were obtained without the addition of exogenous cytokines. This suggests either that CD40 and antigen receptor ligation may be sufficient to induce isotype switching or that bystander cells may provide sufficient cytokines to switch

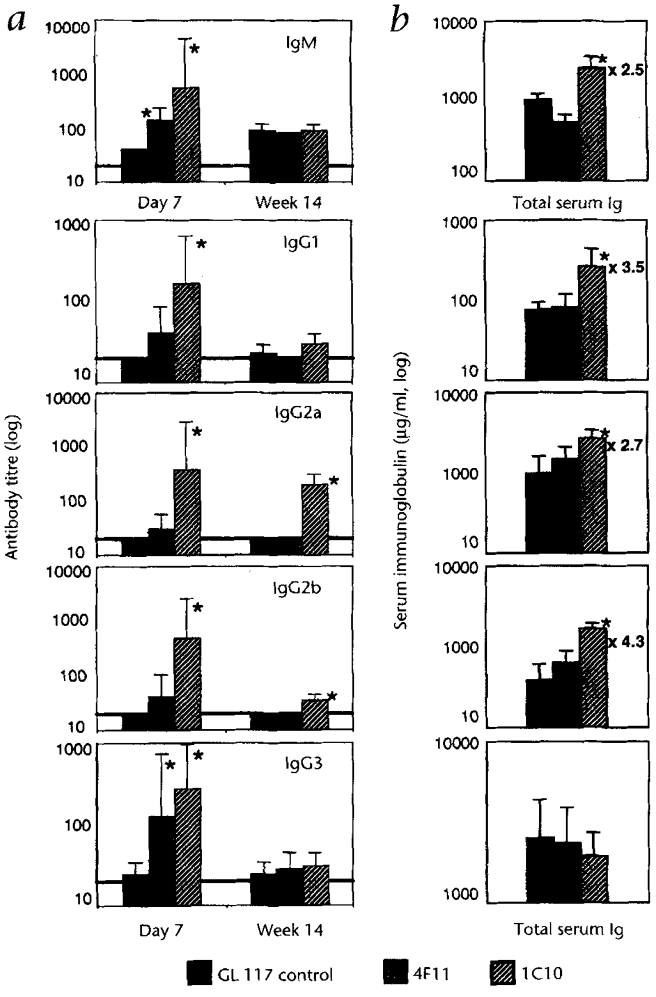


Fig. 1 CD40 antibodies induce ***a***, enhanced class-switched antibody responses to PS3 and ***b***, increased total serum immunoglobulin. Mean logarithmic titers are displayed for serum samples taken, days 7 and 14 and week 14 after BALB/c mice were injected with PS3 and 1C10, 4F11 or isotype control antibody GL117. The IgM and IgG isotype mean logarithmic titers are shown when they were maximal, respectively, days 7 and 14 after injection and also at 14 weeks. All negative results were given a logarithmic titer of 20, the lowest dilution used. Statistical significance compared with the relevant GL117 control by Student's *t*-test, $*P < 0.05$.

the activated B cells *in vivo*[12,14,15] We considered that the CD40 antibodies might be stimulating T-cell cytokine production, either directly through ligation of CD40 on T cells[16,19] or indirectly through induction of costimulatory molecules on B cells or other antigen-presenting cells[17–19]. The 1C10 antibody does induce high levels of costimulatory molecules, whereas 4F11 has no effect (ref. 10 and unpublished data). The action of 4F11 showed T-cell dependency, as it failed to augment polysaccharide-specific responses in CD4-depleted mice (Fig. 3). However, 1C10 and PS3 administration induced a pronounced, isotype-switched response in CD4-depleted mice (Fig. 3), with IgG responses to polysaccharide being better than those induced in normal mice, demonstrating a CD4-independent action. Similar results were obtained when athymic nude mice were used instead of CD4-depleted mice (data not shown).

Most vaccines under development for use against encapsulated bacteria are protein–polysaccharide conjugates, which aim to provide T-cell help for the anti-polysaccharide response through T-cell recognition of epitopes on the protein. By their nature, such conjugates are not as effective in CD4-deficient patients such as those with AIDS (ref. 20, 21). In contrast, the use of a CD40 stimulator would not only avoid the high cost of conjugate production, but as we have shown, would generate responses unaffected by a CD4 deficiency.

The major fault with capsular polysaccharide only vaccines is that infants and young children, while reacting normally to TD antigens, respond poorly to TI type II antigens. Indeed, children under 2 years old fail to respond at all to many TI type II antigens[22–25] The inability of their immune systems to act against bacterial capsules correlates with increased susceptibility to infection. They are the group most in need of effective vaccines. CBA/N (xid) mice have an X-linked immunodeficiency rendering them, like infants, unable to respond to TI type II antigens[26]. Although one report has stated otherwise[27], in our hands, B cells from CBA/N mice react normally to CD40 ligation *in vitro* (ref. 28 and unpublished data A.H.). We immunized groups of xid mice with 1C10 plus PS3 and successfully generated IgG2a and IgG2b responses against PS3 (Fig. 4*a*). Thus, the B-cell defect in these mice was successfully by-passed by administering the CD40 antibody as an adjuvant along with antigen.

Using the mouse model system, we have shown that CD40 stimulators can enhance the antibody response to pneumococcal polysaccharides, producing greater antibody levels and the production of IgG isotypes. As do protein–polysaccharide conjugates, 1C10 can induce polysaccharide-specific responses in xid mice, which like infants are unable to respond to vaccines based on polysaccharide only. Unlike protein–polysaccharide conjugates, the adjuvant action of 1C10 is $CD4^+$ cell-independent, which is a definite advantage for the vaccination of patients with CD4 deficiencies, for example, AIDS sufferers[20,21]. One potential problem with the use of CD40 stimulators as adjuvants may be autoantibody production. We believe the risk should be minimized by improved delivery of antigen and CD40 stimulator. Finally, while 1C10

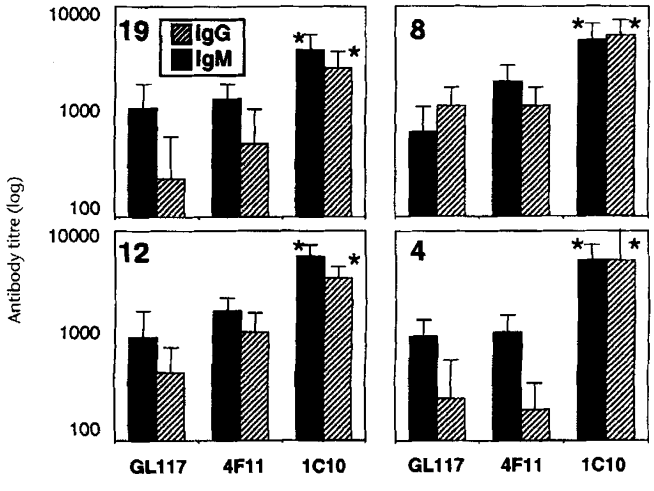


Fig. 2 Antibody responses to other pneumococcal polysaccharides are also enhanced by CD40 antibody. IgM and IgG responses to types 19, 8, 12 and 4. *S. pneumoniae* capsular polysaccharides in mice 10 days after immunization with the 23 capsular polysaccharides in Pneumovax II and either the CD40 antibody 1C10 or control antibody GL117. All the 1C10 responses were significantly different from the GL117 responses by Student's *t*-test, $*P < 0.05$.

Fig. 3 The mechanism of 1C10 action is CD4⁺ T cell-independent. PS3-specific antibody logarithmic titers (day 14) were induced in CD4-depleted BALB/c mice treated i.p. with PS3 and 1C10, 4F11 or control antibody GL117. All 1C10 responses were significantly different from the relevant GL117 control by Student's *t*-test, $^*P < 0.05$.

administered with PS3 clearly enhances specific antibody responses, the measure of a vaccine is whether it provides long-term protection against disease. We challenged mice, immunized 9 months previously, with 10^5 colony-forming units (CFU) of *S. pneumoniae* type 3 (Fig. 4*b*). Of the BALB/c mice administered with PS3 and 1C10, 5 of 8 survived challenge, whereas only 1 of 6 and none of 11 mice survived in the groups receiving, respectively, PS3 with GL117 and PS3 alone (chi-square test, $P < 0.05$).

The CD40 stimulators, such as antibodies, recombinant soluble CD154, or molecular mimics of CD154, have considerable potential as immunological adjuvants for T cell-independent antigens.

Methods

Mice and materials. The mice used were BALB/c mice (in house), CBA/ca and CBA/N (xid) mice (Harlan-Olac UK, Bicester). They were 6–12 weeks old at the start of the experiments. The pneumococcal capsular polysaccharides type 1, 3, 4, 8, 12, 13, 19 and 23 were obtained from American Type Culture Collection (ATCC, Rockville, MD), pneumococcal cell wall polysaccharide from Statens Serum Institut (Copenhagen, Denmark) and Pneumovax II vaccine from Merck, Sharp and Dohme (Hoddesden, UK). The anti-CD40 antibodies, 1C10 and 4F11 (ref. 10), with their isotype-matched rat IgG2a control antibody GL117, were purified by Sheffield Hybridomas (Sheffield, UK).

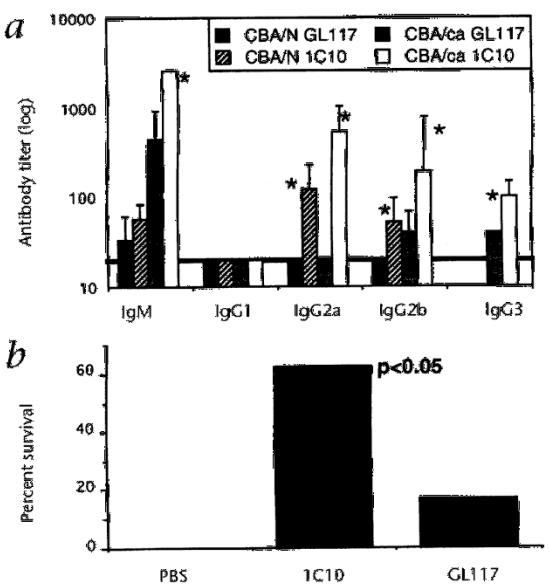
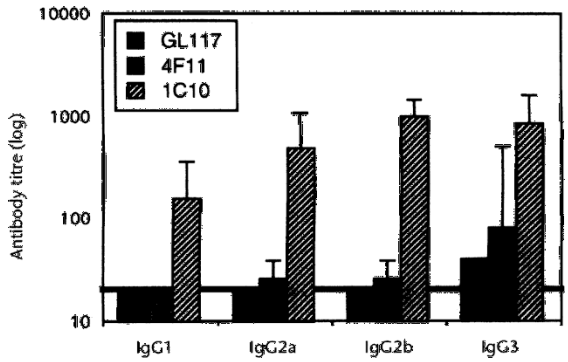


Fig. 4 CD40 antibodies induce responses to PS3 in normally unresponsive xid mice (*a*). Enhanced responses in BALB/c mice provide protection against *S. pneumoniae* challenge 9 months after treatment (*b*). ***a***, PS3-specific antibody responses in CBA/N (xid) mice and control CBA/ca mice injected with PS3 and 1C10 or GL117. The IgM and IgG isotype logarithmic titers shown are when they were maximal, respectively, day 7 and day 14 after injection. Statistical significance compared with the relevant GL117 control (Student's *t*-test, $^*P < 0.05$). ***b***, Percentage survival in BALB/c mice challenged with *S. pneumoniae* type 3, but 9 months previously administered PS3 and 1C10, GL117 or PBS. There were 8, 6 and 11 mice in the 1C10, GL117 and PBS treated groups, respectively. Survival in the 1C10 group was significantly enhanced compared with the control groups (chi-square test, $P < 0.05$).



Immunization protocols. Mice were treated with 500 µg of 1C10, 4F11 or GL117 and 20 ng PS3 i.p., except those receiving Pneumovax II. BALB/c mice receiving Pneumovax II were injected i.p. with 500 µg of either 1C10 or GL117 and 1/25th of the recommended human dose of Pneumovax II. This equates to 1 µg of each of the 23 polysaccharides present in the vaccine. At least five mice were used for each experimental group.

Experiment in CD4-depleted mice. BALB/c mice, 6–10 weeks old, were depleted of CD4 cells 5 days before the start of the experiment. Depleting anti-CD4 antibody YTS 191.1 (500 µg) was injected intravenously and again the next day intraperitoneally. The percentage of CD4⁺ splenocytes in the depleted mice as detected by flow cytometry had dropped to undetectable levels when the antibody and PS3 were injected. There was no IgG antibody response of these mice to 50 µg of keyhole limpet hemocyanin, a T cell-dependent antigen (data not shown).

Measurement of polysaccharide antibodies and total serum immunoglobulin by ELISA. First, 96-well ELISA plates (Costar, High Wycombe, UK) were coated overnight with 10 µg/ml polysaccharide or with a 1/200 dilution of anti-mouse immunoglobulin serum (Sigma, Poole, UK). Individual serum samples were titrated on the plates, and the various isotypes were detected by horseradish peroxidase (HRP)-conjugated mouse isotype-specific serum (Southern Biotechnology Associates, Birmingham, AL). Serum obtained from mice injected with Pneumovax II was absorbed against *S. pneumoniae* cell wall polysaccharide as described previously[29]. Antibodies to cell wall polysaccharide, a contaminant of all capsular polysaccharide preparations, might have created false-positive results. Total serum immunoglobulin concentrations were calculated with reference to calibrated mouse serum (Sigma). With the polysaccharide results end-point titers for each mouse were assessed against normal mouse serum, and then logarithmic mean titers and standard deviation were calculated. All negative results were given a logarithmic titer of 20, the lowest dilution used.

Challenge with *S. pneumoniae*. BALB/c mice were immunized 9 months before challenge with 20 µg PS3 and 500 µg 1C10 i.p. Challenge was 10^5 CFU of encapsulated *S. pneumoniae* type 3 (ATCC) given i.p. Numbers of surviving animals were ascertained 2 weeks after challenge.

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