

RELATIONSHIP BETWEEN CYTIDINE 5'DIPHOSPHOCHOLINE EPI TOPE DENSITY ON OVALBUMIN AND IgE ANTIBODY RESPONSES COMPARED TO OTHER ISOTYPES

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Résumé

RELATION ENTRE LE DEGRÉ DE SUBSTITUTION DE L'OVALBUMINE PAR LA CYTIDINE 5'DIPHOSPHOCHOLINE ET LA RÉPONSE EN ANTICORPS IgE. — L'ovalbumine a été substituée covalentiellement par 6, 18 ou 20 résidus de Cytidine 5'diphosphocholine par molécule. Après injection à des souris BALB/c, ces produits induisent des réponses immunitaires distinctes. Sans résidu ou avec six résidus, l'ovalbumine induit des anticorps hémagglutinants (1/256, 21 jours après injection) et une réponse à la réaction anaphylactique cutanée passive (1/400, 21 jours après injection). Par contre, avec 18 ou 20 résidus, l'ovalbumine provoque seulement l'apparition d'un faible titre d'anticorps hémagglutinants (1/64, 20 jours après injection) ; aucune réponse à la réaction anaphylactique cutanée passive n'est observée. Aucun anticorps anti-cytidine 5'diphosphocholine n'a été détectée par l'une ou l'autre réaction. Il apparaît donc que la densité en épitopes (déterminants antigéniques) joue un rôle essentiel dans la réponse immune à IgE anti PC-OA.

Conformational and sequential determinants on an antigenic molecule were shown to play a role in the immune response (Sela, 1969). Depending upon its conformation, the same antigen can be either tolerogenic or immunogenic (Dresser *et al.*, 1968) ; for instance succinylation, citraconylation or acetylation of a protein (Parish, 1971) transforms an immunogenic molecule into a tolerogenic one.

Recently, IgE suppression has been obtained for anti-carrier-directed IgE antibodies. Polyethylene glycol coupled with bovine liver catalase induced a loss of immunogenicity of the carrier molecule (Abuchowaski *et al.*, 1977) and coupled with ovalbumine (OA) or with ragweed pollen it formed poor immunogenic compounds which were able to tolerize mice against either OA or ragweed ; moreover these compounds

suppressed an on-going IgE response against either OA or ragweed (Lee and Sehon, 1978). Preadministration of 100 µg of OA conjugated muramyl dipeptides partially inhibited the primary and secondary induction of anti-OA IgE antibody response, while anti-OA IgE antibody response was about the same as that of control mice when tested by transferred cells (Kishimoto *et al.*, 1979). Conjugates of proteins and protein D-glutamic acid-D lysine reproducibly induce significant unresponsiveness to the protein antigens in experimental mice ; this unresponsive state could be induced in both unsensitized and previously sensitized mice. It was confined to responses of the IgE antibody class and was highly antigen-specific (Liu *et al.*, 1979). It is noteworthy that in all these reported experiments the same high level of tolerance

was induced by extremely high doses of antigens (100 μ g to 1 mg).

However, none of these experiments mentioned a possible role of the epitope density on the immune response against the carrier molecule. As a general rule, increasing the number of hapten molecules grafted on the carrier is assumed to lower the immune response : for instance, guinea-pigs immunized with lowly substituted dinitrophenyl-bovine gamma globulin produced large amounts of antibody directed against the carrier and large amounts of antibody directed against the hapten ; guinea pigs immunized with a highly conjugated preparation did not produce antibody against the carrier but produced large amounts of antibody against the hapten (Quijada *et al.*, 1974).

In order to immunize against ovalbumin, while avoiding the IgE antibodies and/or suppressing them against OA, we investigated the role of the number of phosphorylcholine residues to be grafted. We found that the injection of highly substituted ovalbumin induces a low hemagglutinating antibody and no IgE antibody response.

Materials and Methods

Animals

Inbred 4 to 6-week-old BALB/c mice and 4-month-old Lewis female rats (350 g) were bred in our animal colony.

Chemicals

The 5-times recrystallized ovalbumin (OA) was purchased from Calbiochem (La Jolla, USA) and *Limulus polyphemus* hemocyanin from Sigma (St-Louis, USA). Cytidine 5'diphosphorylcholine (PC) was obtained from Boehringer (Mannheim, Germany). All other chemicals used were of analysis grade.

Preparation of hapten carrier conjugates

The detailed method for the preparation of PC-human serum albumin and PC-hemocyanin conjugates with various epitope densities is described by Pery *et al.* (1979). From our previous data it was inferred that by varying the amounts of oxidized PC in the reaction mixture, conjugates with different substitution degrees

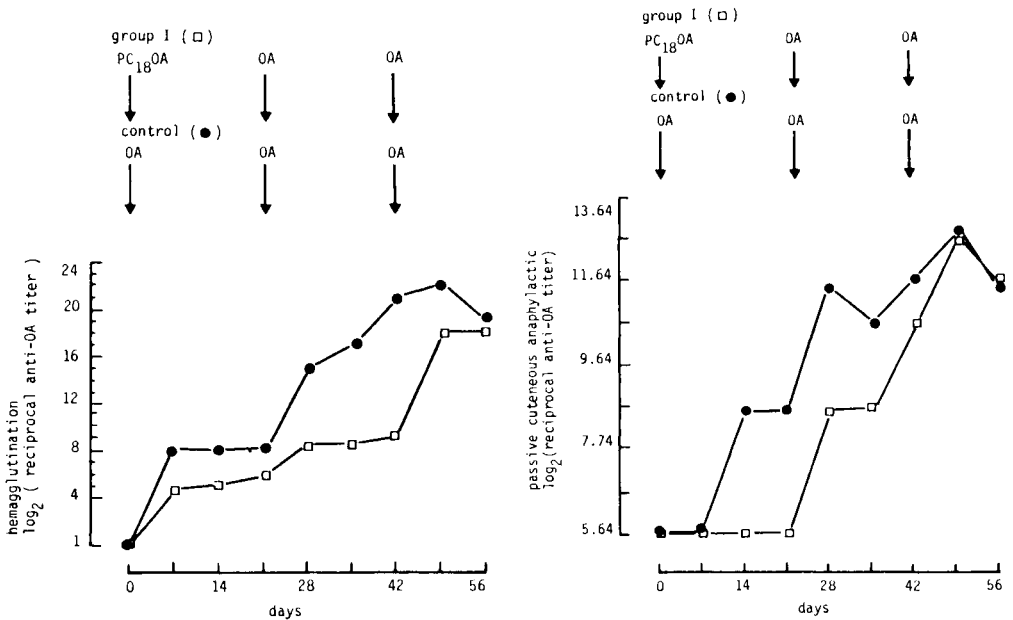


Fig. 1. — The primary and secondary immune response of mice injected either with OA or with only one injection of PC 18 OA.

a : as measured by hemagglutination

b : as measured by passive cutaneous anaphylactic reaction. (In passive cutaneous anaphylactic reaction, first dilution 1/50 : 5.64 in \log_2 .)

might be obtained. Thus 20 or 25 μM oxidized PC were reacted with 2.0 μM OA: estimation of the substitution extent from the respective absorbencies at 272 and 280 nm of conjugates were consistent with averages of 18, 20 and 6 PC residues respectively per molecule of conjugate. These conjugates will be described as PC 6 OA, PC 18 OA and PC 20 OA.

Adjuvant

AlPO_4 gel was prepared by mixing 0.2 M AlCl_3 and 0.2 M Na_3PO_4 , pH 5, the final concentration being 10 mg/ml according to Holt (1969). Adjuvant and antigen were mixed 1 to 1 (v/v) and stirred for 1 h at room temperature before injection

Immunizations and bleedings

Ten mice per group were immunized intraperitoneally with 2 μg of antigen absorbed on 1 mg of AlPO_4 gel on days 0, 21 and 42. Bleedings

were performed from the retro-orbital sinus and sera were pooled in each experimental group and stored at -20°C .

Titration of antibodies

— Passive hemagglutination test

Hemagglutinating antibodies were measured by agglutination of either OA or PC-hemocyanin-coated sheep red blood cells (Lemieux *et al.*, 1974) on microplates (Limbro Scientific Co.).

— Passive cutaneous anaphylactic reaction

IgE antibodies were titrated by heterologous passive cutaneous anaphylactic reaction (PCA) on rats as described by Watanabe *et al.* (1977). In short, 0.2 ml of antiserum dilutions were injected intradermally into three rats (five dilutions per animal) for each pool. Twenty-four hours later 2 mg of antigen was injected intravenously (OA to detect anti OA antibodies, PC-hemocyanin to detect PC antibodies). PCA reaction was checked 30 min after the antigen injection.

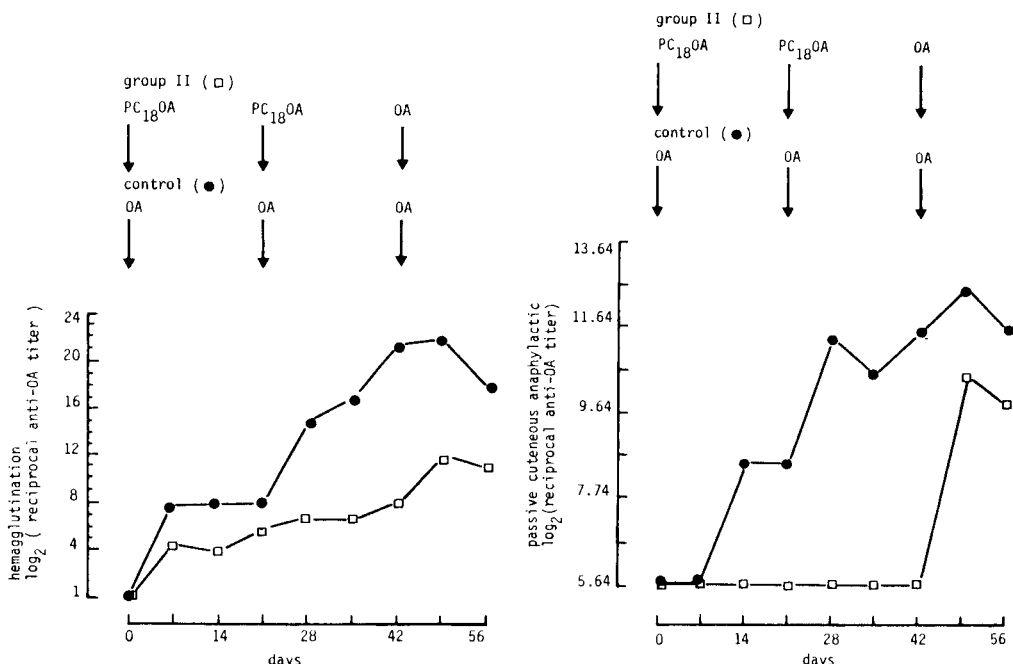


Fig. 2. — The primary and secondary immune response of mice injected either with OA or with two injections of PC 18 OA.
 a : as measured by hemagglutination
 b : as measured by passive cutaneous anaphylactic reaction. (In passive cutaneous anaphylactic reaction, first dilution 1/50 : 5.64 in \log_2 .)

Table 1. — Comparison of primary responses of BALB/c mice to ovalbumin and substituted ovalbumin with passive hemagglutination and passive cutaneous anaphylaxis

No. of residues per molecule of ovalbumin	Antiovalbumin reciprocal titers					
	Hemagglutination			Passive cutaneous anaphylaxis		
	7	days 14	21	7	days 14	21
0	256	256	256	<50	200	400
6	256	256	256	<50	200	400
18	32	16	64	<50	<50	<50
20	32	16	64	<50	<50	<50

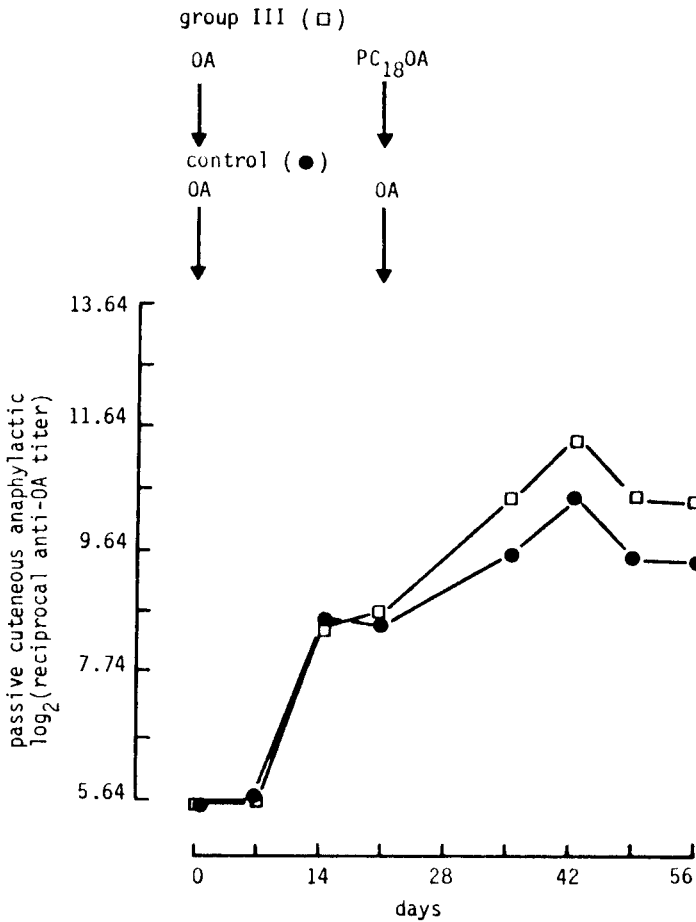


Fig. 3. — Failure to suppress an on-going IgE response by one injection of PC 18 OA as measured by passive cutaneous anaphylactic reaction. (First dilution 1/50 : 5.64 in log₂.)

Results

Hemagglutinating antibodies are considered to be of IgM and IgG isotypes while PCA antibodies are of IgE isotype. During the whole experiment we did not find any anti-PC antibodies, all antibodies being directed against the carrier.

The primary anti-OA response (table 1)

The PC6OA antigen induced hemagglutinating antibodies and PCA antibodies as OA did ; on the 3rd week after immunization the HA titer reached 1/256 while the PCA titer attained 1/400. In contrast, PC 18 OA or PC 20 OA induced a lower HA antibody titer (1/32 on the third week) and no IgE as tested by PCA.

The secondary anti-OA response

In group I, mice receiving PC18OA, and thereafter two booster injections of OA, exhibited a slightly lower secondary response than controls as measured by hemagglutination (fig. 1a). IgE measured by PCA (fig. 1b) was undetectable for three weeks and appeared only seven days after OA injection at a lower titer than in the control group. However, PCA titers were the same in both experimental and control group after the second booster injection of OA on day 42 (fig. 1b). Similar results were obtained with PC20OA (data not shown).

In group II, for mice receiving twice PC18OA and a booster injection of OA, HA titers were always below that of control animals. Thus PC18OA was shown to be less immunogenic than OA in relation to the OA immune response

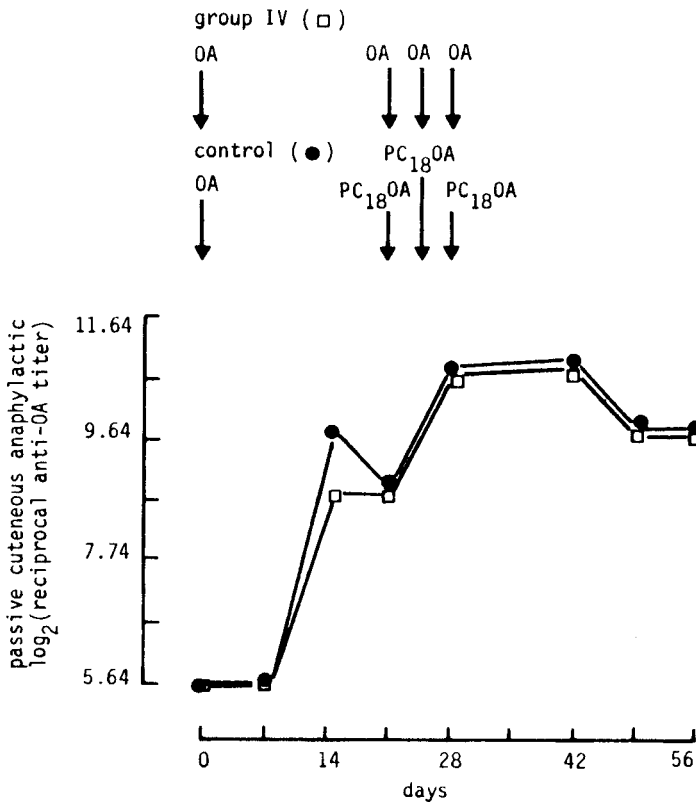


Fig. 4. — Failure to suppress an on-going IgE response by three injections of PC 18 OA as measured by passive cutaneous anaphylactic reaction. (First dilution 1/50 : 5.64 in \log_2 .)

(fig. 2a). PCA antibodies were undetectable during six weeks until unsubstituted OA was injected (fig. 2b). Then mice exhibited a true secondary response characterized by a peak on the seventh day after OA injection (fig. 2b). Mice injected with PC20OA behaved similarly. Thus PC18OA or PC20OA were found unable to induce any IgE response. However, sensitized animals behaved as primed animals when OA was injected. Meanwhile, IgE titers stayed below those of control animals.

Failure to suppress the on-going IgE response

Either one (group III, fig. 3) or three (group IV, fig. 4) injections of PC18OA delivered three weeks after a primary immunization with OA induced the same secondary response as OA alone.

Discussion

This study shows that chemical coupling of PC with ovalbumin modulates the anti-carrier immune response. While conjugation of six PC residues to ovalbumin does not significantly alter the overall immunogenicity or antigenicity of the protein, conjugation of 18 or 20 residues to the molecule leads to a decrease of immunogenicity, which is complete for the induction of a primary IgE immune response and only partial for other antibody responses. A simple denaturation of OA during the coupling treatment is not involved in this inhibition since PC6OA is as immunogenic as OA alone. In more recent experiments PC14OA was found to exhibit the same properties as OA and PC6OA. It is well known that grafting haptens onto a carrier molecule as described in this presentation leads to heterogenous populations of molecules, so that the indicated number of hapten residues per molecule of the carrier represents an average. It is therefore striking to see the difference occurring between PC14OA and PC18OA in immunogenic properties.

A selective inhibition of IgE response against various antigens has been obtained by conjugating antigens to high molecular weight compounds: polyethylene glycol (Lee *et al.*, 1978; Sehon, 1979) or random copolymers of D-glutamic acid and D-lysine (Liu *et al.*, 1979).

True hapten-carrier systems give less clear-cut results. Quijada *et al.* (1974) showed that guinea-pigs immunized with lowly substituted dinitrophenylated bovine gamma globulin produce antibodies against both the hapten and

the carrier molecules. By increasing the substitution degree of the protein, they noted a progressive decrease of the anti-carrier antibody response, with a persistence of the anti-hapten response. Lee and Sehon (1976) evidenced a similar relationship between the epitope density and the immunogenicity of haptenated ovalbumin for hemagglutinating and reaginic antibodies. In a recent study Nakagawa *et al.* (1980) were unable to show any significant difference in the carrier specific IgE immune response induced by moderately and highly substituted conjugates. The use of different haptens may perhaps explain some of these discrepancies.

In our experiments, we could not find any anti-hapten antibodies. This fact may be related to the peculiar anti-phosphorylcholine antibody response of the BALB/c mouse (Gearhart *et al.*, 1977). The relevance of the decreased allergenicity of PC18OA and PC20OA with this absence of anti-PC response is unknown.

The decrease in allergenicity of the carrier molecule when PC residues are increased can be explained either by the overall epitope density or by the existence of a specific target on OA, which is reached only with high hapten to carrier ratio. During our coupling procedure, PC was bound to lysyl residues of protein (OA contains 20 lysyl). It would be interesting to bind the same hapten onto another amino-acid (i.e. tyrosine) to demonstrate the importance of a possible specific link between the hapten and the carrier. The use of related hapten (i.e. cytidyl residues) would enable us to define more precisely the role of the hapten in this phenomenon.

With such a low antigen dose we were unable to inhibit an on-going IgE response induced by OA alone. By contrast, a state of tolerance specifically directed against the IgE isotype was obtained with very high doses (from 10 μ g to 1 mg) by injections of modified antigens either of muramyl-dipeptide (Kishimoto *et al.*, 1979), or copolymer of D-glutamic acid and D-lysine (Liu *et al.*, 1979) or of polyethylene glycol (Sehon *et al.*, 1979). Moreover, in our experiments one or two injections of PC18OA followed by one injection of OA led to a typical secondary response in IgE anti-OA antibodies, though it reached lower titers than in controls. Work is in progress to see if the complete molecule is required in cytidine diphosphocholine, or if cytidine triphosphate might play the same role.

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Summary

Ovalbumin was covalently substituted by 6, 18 and 20 residues of Cytidine 5'diphosphocholine per molecule. When injected into BALB/c mice, these products induced distinct immunogenic responses. Without any residue, or with six residues, ovalbumin induced hemagglutinating antibodies (1/256, 21 days after injection) and a response to the passive cutaneous anaphylactic reaction (1/400, 21 days after injection). By contrast, with 18 or 20 residues, ovalbumin brought about the appearance of only a weak titer of hemagglutinating antibodies (1/64, 21 days after injection) ; no response to the passive cutaneous anaphylactic reaction was observed. No anti-cytidine 5'diphosphocholine antibodies were detected by either reaction. Thus epitope density plays a major role in the IgE immune response to PC-OA.

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