

Short Communication

Adjuvant Effect of Benzalkonium Chloride on the Allergen-Specific IgE, IgG1 and IgG2a Antibody Formation in BALB/cJ Mice

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The quaternary ammonium compounds, of which benzalkonium chloride is the most frequently used, are cationic surfactants used e.g. as antiseptics, disinfectants, detergents and preservatives (Purohit *et al.* 2000). Quaternary ammonium compounds may possess adverse effect on the immune system. Thus, atopic sensitization, defined as increased IgE levels to common aeroallergens, occurred more frequently in pig farmers who used disinfectants containing these compounds – an effect which the authors suggested could be due to an adjuvant effect of the used compounds (Preller *et al.* 1996; Vogelzang *et al.* 1997). In itself, benzalkonium chloride seldomly causes allergic airway diseases (Purohit *et al.* 2000).

The adjuvant effects on antibody production of several quaternary ammonium compounds have been revealed in a guinea pig study (Gall 1966), however not differentiated in antibody isotypes. Sparse information is available about the adjuvant effect of benzalkonium chloride. The present study investigates the effect of benzalkonium chloride on the murine type 1 helper cells (Th1) and Th2-dependent antibody levels. The Th1 response elicits IgG2a antibodies, whereas the Th2 response promotes formation of IgE and IgG1 (Corry & Kheradmand 1999).

The activity of one of the most potent quaternary ammonium compound adjuvants, dioctadecyldimethylammonium bromide (DDA), has been shown in several studies to be influenced by other substances (Hilgers & Snippe 1992). As commercial quaternary ammonium compound-based products may contain a mixture of many quaternary ammonium compounds, we studied a possible interaction between benzalkonium chloride and tetraethylammonium bromide, a quaternary ammonium compound, which not *per se* possesses adjuvant properties as it contains short al-

kyl chains (Gall 1966) as confirmed by us (Larsen *et al.* 2004).

Female BALB/cJ mice (Bomholtgård Breeding and Research Center, Denmark), housed as previously described (Larsen *et al.* 2004), were immunised by subcutaneous injection of the model allergen ovalbumin (CAS 9006-59-1, purity >98% (Grade V)) in the neck region with or without benzalkonium chloride (predominantly benzyldodecyldimethylammonium chloride, CAS 8001-54-5) and tetraethylammonium bromide (CAS 56-34-8, purity >98%). Ovalbumin, benzalkonium chloride and tetraethylammonium bromide were all from Sigma-Aldrich Chemie, Steinheim, Germany. DDA (CAS 3700-67-2, purity >99%) from ABCR Chemicals, Karlsruhe, Germany and aluminium hydroxide (Al(OH)₃, Alhydrogel® 2%) from Brenntag Biosector, Frederikssund, Denmark were used as positive adjuvant controls. Details of the sensitisation protocol are given in tables 1 and 2. The level of ovalbumin-specific IgE, IgG1 and IgG2a antibodies in the serum were determined by means of ELISA (Larsen *et al.* 2002).

The lowest dose of benzalkonium chloride, 0.1 µg, showed a statistically significant adjuvant effect on the IgE production after one booster injection (table 1). There was a significant adjuvant effect of benzalkonium chloride on the IgG1 response both after one and two booster injections (table 1). After one booster the adjuvant effect was apparent at the 0.1, 1, and 100 µg dose levels but surprisingly not at the 10 µg level. After the second booster, the adjuvant effect was apparent from the two lower doses of benzalkonium chloride.

Gall (1966) found no important adjuvant effect of 100 µg benzalkonium chloride when investigated by subcutaneous administration of diphtheria toxoid in guinea pigs followed by two booster injections. Nor did we observe any statistically significant adjuvant effect after administration of 100 µg benzalkonium chloride and two booster injections. Higher doses of benzalkonium chloride occasionally suppressed the IgE and IgG1 antibody formation (tables 1 and

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Table 1.

Formation of ovalbumin (OVA) specific IgE and IgG1 antibodies after subcutaneous injection of 1 µg OVA alone (0 µg BAC) or 1 µg OVA together with benzalkonium chloride (BAC) in 100 µl saline. Animals were immunised on day 0 and boosted subcutaneously with 0.1 µg OVA in 100 µl saline on day 14 (1 booster animals) or on days 14 and 21 (2 booster animals). Blood was collected either on day 28 (1 booster animals) or on day 35 (2 boosters animals). The Al(OH)₃ and dimethyldioctadecylammonium bromide (DDA) groups followed the same time schedule as the BAC groups.

	Dose of BAC (µg)					Positive adjuvant control groups		
	0	0.1	1	10	100	270 µg Al(OH) ₃	250 µg DDA	
IgE 1 booster	120 (79–158) ^a	199 (96–256) 1.7 ^{b,c}	150 (97–301) 1.3	86 (27–167) 0.71	49 (30–375) 0.41	233 (101–401) 1.9 ^{**}	302 (220–385) 2.5 ^{***}	
IgG1 1 booster	32 (15–42)	80 (32–306) 2.5 ^{***}	136 (38–407) 4.3 ^{***}	31 (0–63) 1.0	871 (46–2876) 28 ^{***}	399 (106–2210) 13 ^{***}	1716 (817–7801) 54 ^{***}	
IgE 2 boosters	682 (394–1567)	1033 (596–1650) 1.5	827 (474–2489) 1.2	277 (159–479) 0.41 ^{***}	251 (183–333) 0.37 ^{***}	972 (729–1326) 1.4	539 (281–940) 0.79	
IgG1 2 boosters	562 (235–1295)	1045 (473–5306) 1.9 ^{**}	1566 (489–6720) 2.8 ^{**}	247 (105–1355) 0.44	666 (121–1669) 1.2	33528 (2366–68924) 60 ^{***}	14402 (2168–43654) 26 ^{***}	

^a Median antibody level (arbitrary unit) of the group of mice (n=20–39) with the 25 and 75% percentiles in parenthesis

^b Ratio between antibodies in the exposure group and in the group immunised by OVA alone

^c Level of significance from a pair-wise comparison between the exposure group and the OVA control group by means of the Mann-Whitney's U-test. *P<0.05, **P<0.01, ***P<0.001.

2), an effect seen previously from higher doses of other surface-active substances (Clausen *et al.* 2000). No IgG2a antibody formations were seen in any of the animals in the ovalbumin control or benzalkonium chloride exposure groups (data not shown). However, with other test substances the applied sensitization protocol has resulted in production of IgG2a antibodies (Larsen *et al.* 2002 & 2004).

The interaction experiment showed that the adjuvant effect of benzalkonium chloride on IgG1 after one booster injection disappeared after addition of 500 µg tetraethylammonium chloride to the benzalkonium chloride/ovalbumin solution (table 2). In addition, the suppressive effect of benzalkonium chloride seen on the IgG1 production after 2 boosters was reduced in the presence of tetraethylammonium chloride. Thus, with respect to IgG1, tetraethylammonium chloride counteracted the adjuvant effect as well as the suppressive effect of benzalkonium chloride, whereas tetraethylammonium chloride had little effect on the IgE antibody production.

The study by Gall (1966) showed that benzalkonium chlorides with 16 or 18 carbon atoms in the alkyl side chain are stronger adjuvants than benzalkonium chloride with 12 carbon atoms, which was used in this study. We showed that benzalkonium chloride with a 12 carbon atoms long alkyl chain is a potent enhancer of Th2 cytokine dependent antibodies. Hence, a very low dose of benzalkonium chloride (0.1 µg) gave rise to IgE enhancement comparable to that seen after administration of 270 µg Al(OH)₃, and thus, benzalkonium chloride is much more potent than Al(OH)₃ to stimulate the IgE production. Benzalkonium chloride also enhanced the production of IgG1, another Th2 cytokine dependent antibody. As IgE is the more relevant antibody with respect to type I allergy, risk assessment of a substance should be based on its ability to stimulate the IgE production rather than the IgG1 production.

In the present study the subcutaneous route of administration was used to identify a possible hazard in the airways. This can be justified as different routes of administration of an allergen in combination with an adjuvant gave rise to the same outcome (Larsen *et al.* 2004).

Our screening study intends to distinguish between substances with and without adjuvant effect. Risk assessment has to be based on the relevant exposure route, which for inhalation studies are much more costly and, thus, for cost-effectiveness should be limited to substances selected from screening studies.

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Table 2.

Formation of ovalbumin (OVA) specific IgE and IgG1 antibodies after subcutaneous injection. BAC: benzalkonium chloride, TEA: tetraethylammonium bromide, DDA: dimethyldioctadecylammonium bromide. Immunisations were with OVA: 1 µg, BAC: 100 µg, TEA: 500 µg, Al(OH)₃: 270 µg and DDA: 250 µg on day 0. Animals were boosted subcutaneously with 0.1 µg OA in 100 µl saline on day 14 (1 booster animals) or on days 14 and 21 (2 booster animals). Blood was collected either on day 28 (1 booster animals) or on day 35 (2 boosters animals). The Al(OH)₃ and DDA served as positive adjuvant control groups and followed the same time schedule as the BAC groups.

	OVA	OVA + BAC	OVA + BAC + TEA	OVA + Al(OH) ₃	OVA + DDA
IgE 1 booster	121 (66–149) ^a	36 (0–44) 0.30 ^{b***c}	15 (0–46) 0.12 ^{***}	296 (159–385) 2.4 ^{**}	397 (305–1350) 3.3 ^{***}
IgG1 1 booster	37 (30–59)	110 (47–5963) 3.0 [*]	40 (23–65) 1.1	2141 (1764–2819) 58 ^{***}	2163 (1416–5653) 58 ^{***}
IgE 2 boosters	630 (302–1720)	209 (160–323) 0.33 ^{**}	168 (98–215) 0.27 ^{**}	814 (710–996) 1.3	281 (146–558) 0.45 [*]
IgG1 2 boosters	372 (173–657)	112 (48–190) 0.30 ^{**}	338 (54–642) 0.91	2174 (833–44095) 5.8 ^{***}	2475 (2096–16640) 6.7 ^{***}

^a Median antibody level (arbitrary unit) of the group of mice (n=10–20) with the 25 and 75% percentiles in parenthesis.

^b Ratio between antibodies in the exposure group and in the control sensitised by OVA alone.

^c Level of significance from a pair-wise comparison between the exposure group and the OVA control group by means of the Mann-Whitney's U-test. *P<0.05, **P<0.01, ***P<0.001.

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