

The adjuvant activity of *Bacillus subtilis* and subtilisin

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B. subtilis cells were found capable of adjuvant activity in producing anaphylaxis in the mouse when administered together with antigen via the footpad route. Subtilisin also exhibited an adjuvant effect, which was evident at the time of secondary challenge. The spores were ineffective. Skin-sensitizing antibodies were present in all the sera as demonstrated by the 2 hour passive cutaneous anaphylaxis technique, even though the animals failed to exhibit anaphylactic reactions. Precipitating antibodies to subtilisin were demonstrated in the sera of mice prepared by the adjuvant action of *B. pertussis* with cells, spores and subtilisin. These animals were not anaphylactically sensitive.

Our¹ early experiments in the inherently resistant mouse have shown that an enhanced anaphylactic sensitivity results following the simultaneous intraperitoneal inoculation of *Bordetella pertussis* phase I cells and a protein antigen. Of the vaccines studied, only that of *Brucella abortus* exerted a similar effect, though to a lesser degree.^{2, 3} Because of the possibility of a clinical sensitization resulting from inhalation of *Bacillus subtilis* and/or its derivatives,^{4, 5} it was considered of interest to investigate the adjuvant capacity of these agents.

MATERIALS AND METHODS

Mice

Carworth Farm (CFW) mice of mixed sexes were used exclusively. The animals weighed about 25 grams at the start of the experiments and were kept in segregated groups maintained on Purina Laboratory Chow and water ad libitum.

Adjuvants

*B. subtilis** was cultured on agar slants enriched with Difco Heart Infusion Broth. The cells were harvested after suspension in 0.85 per cent saline solution (SS) and killed by the addition of thimerosal to a concentration of 1:10,000. Spores were purchased from Difco Laboratories as "Subtilis Spore Suspension." Subtilisin was purchased from Sigma Chemical Company and was received labeled "Protease, bacterial, type VII, crystallized and lyophilized." *B. pertussis* phase I cells were taken from our stock used in previous studies.

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Antigen

Crystalline egg albumin (EA) was prepared in this laboratory from fresh hen eggs according to the procedure outlined by Kabat and Mayer.⁶ EA was one of the earliest used proteins in quantitative immunochemical investigations.⁷

Sensitization

Each mouse of a group received one of the following:

1. *B. subtilis* (BS). EA, 0.5 mg. dissolved in 0.03 ml. of the bacterial suspension containing 3×10^7 cells was inoculated into each hind footpad.
2. *Spores*. EA, 0.5 mg. dissolved in 0.03 ml. of the original spore suspension concentrated threefold, was injected into each hind footpad.
3. *Subtilisin*. Each hind footpad was injected with 0.03 ml. of a SS containing 0.5 mg. of enzyme and 0.5 mg. of EA.
4. *B. pertussis* (BPV). A mixture of 0.5 mg. of EA and 3×10^9 BPV cells in 0.03 ml. volume was injected into each hind foot as a reference standard.⁸
5. *Control*. Injections into the hind footpads consisted of 0.03 ml. of a solution containing 0.5 mg. of EA only.

Anaphylaxis

Ten days after sensitization, the mice were bled via the retro-orbital plexus, the expressed serum removed and stored in the frozen state until required for later use. Following the bleeding, 1 mg. of EA per 0.1 ml. of SS was administered to each mouse intravenously as the challenge dose. A similar intravenous secondary challenge dose was administered in most cases to the survivors 7 days later, following an additional bleeding.

Immunodiffusion

Sera from the above bleedings were examined for the presence of precipitating antibodies by the agar gel plate technique of Ouchterlony⁹ against EA in concentrations ranging from 20 mg. per milliliter to 0.625 mg. per milliliter.

Passive cutaneous anaphylaxis (PCA)

The presence of skin-sensitizing antibody was determined by the PCA technique of Ovary¹⁰ using mice as the acceptor animals. The sera from each individual group were pooled, and a volume of 0.05 ml. was injected intradermally into the dorsal surfaces of 3 mice. After a period of either 2 or 48 hours, 1 mg. EA in 0.5 ml. of a 1:10 dilution of Evans blue dye (USP, Warner-Chilcott) was given intravenously. The skins were everted after 15 minutes and the diameters of the blue areas recorded.

RESULTS AND DISCUSSION

B. subtilis was found to be an effective adjuvant in that anaphylactic sensitivity was enhanced. This effect was particularly striking following the secondary challenge, as seen in Table I. Neither the spore- nor the enzyme-treated animals showed any reactions following the primary challenge; a weak sensitivity in the spore-treated and a strong sensitivity in the enzyme-treated mice were demonstrated by secondary challenge.

The ability of the enzyme, subtilisin, to act as an adjuvant is of interest, although the mechanism of action can only be conjectured to be related to the inflammatory reaction seen at the site of the inoculation. In less than an hour, the footpads turn hemorrhagic and become markedly edematous. There may be bleeding from the injection sites. The edema persists and, eventually, a necrotic reaction occurs which results in a slough of the necrotic tissues in-

TABLE I. Anaphylactic sensitization to egg albumin (EA)

Challenge with EA	Sensitized with:				Saline + EA
	B. Subtilis + EA	Spores + EA	Subtilisin + EA	B. pertussis + EA	
Primary at 10 days	7/30*	0/10	0/10	24/29	0/10
Secondary at 17 days	19/23	2/10	6/10	—	1/10

Sensitization accomplished by injection via the footpads; challenge with 1 mg. of EA via the intravenous route.

*Number of animals dead after 2 hours per number of animals challenged.

cluding toes. Healing had not set in by the time for the termination of the experiment. Inflammation with drainage into the regional lymph nodes has been said to be a necessary prerequisite for classical adjuvance.¹¹

Although anaphylactic sensitization was enhanced by the use of *B. subtilis* and subtilisin apparently acting as adjuvants, no precipitins were demonstrable by gel immunodiffusion in any of the sera throughout the range of antigen concentrations employed. This was not an entirely unexpected observation, for *B. pertussis*, a known potent adjuvant, also fails to stimulate circulating precipitins, at least as detectable by the above technique.

Notwithstanding the apparent absence of precipitating antibodies, skin-sensitizing antibodies to EA were found in the sera obtained at secondary challenge of all three groups of mice, that is, those inoculated with EA and utilizing BS, spores, or subtilisin as the adjuvant. These antibodies were demonstrable at 2 hours but not after 48 hours of incubation, producing areas of bluing measuring from 5 × 11 mm. to 25 × 30 mm. In the mouse such antibodies belong to the IgG₁ class. It is to be noted especially that these antibodies were found also in the pool of serum from animals receiving the spores as adjuvant; not only did these animals not have any precipitating antibodies in their sera, but they failed to show any appreciable anaphylactic reactions. None of the pools of sera obtained at the time of primary challenge had any demonstrable skin-sensitizing antibodies. The sera of the BPV-EA-treated mice obtained at the time of primary challenge showed the presence of skin-sensitizing antibodies at the time of the primary challenge; the potent adjuvancy of BPV has been alluded to above. Control sera obtained before primary and secondary challenge were negative throughout. Heating the positive sera at 56° C. for 30 minutes failed to diminish the PCA reaction. IgG₁ is not heat labile.

It was also deemed of interest to determine whether BPV could enhance anaphylactic sensitivity to BS or its derivatives. Consequently, a series of experiments was performed according to the above described techniques wherein BPV (6 × 10⁹ cells) as adjuvant was admixed with BS (6 × 10⁷ cells), spores (0.06 ml. of the original suspension concentrated threefold) or 1 mg. of subtilisin and injected into the footpads. None of the sera obtained at primary challenge (1.5 × 10⁶ cells BS, 0.1 ml. of original spore suspension or 1 mg. of subtilisin) showed the presence of precipitins to suspensions of the spores or cells by gel immunodiffusion. With the use of subtilisin as the antigen (10 mg.

per milliliter), however, 4 distinct bands were observed for each of the three antisera. The enzyme, as expected, behaves as a potent antigen, with the small quantity present in the cells and spores a sufficient stimulus for the production of precipitins. It is important to emphasize that none of the mice showed anaphylactic reactions to the intravenous challenge with enzyme or the other antigens. Thus anaphylaxis in the mouse is not predicated upon the need for circulating, precipitating antibody.

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