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EXPERIMENTAL STUDY OF EVANS' BLUE AS ADJUVANT FOR INDUCING DELAYED HYPERSENSITIVITY

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To induce delayed-type hypersensitivity (DTH) to soluble protein antigens experimentally, an oily adjuvant containing various microorganisms (BCG, Mycobacterium tuberculosis, M. butyricum, etc.) is usually used. Some adjuvants can only intensify developing DTH [3]. Recently much attention has been paid to water-soluble natural and artificial polyelectrolytes, which considerably stimulate the immune response, including DTH [4]. Crowle et al. [5, 6] suggested using the dye Evans' blue (EB) to induce DTH, because it is free from toxicity, can bind with blood proteins, and is used clinically to determine the circulating blood volume [1, 8]. Crowle and co-workers showed that 2-7 weeks after separate injections of EB and antigen into mice the animals developed hypersensitivity which, in its histologic picture and in the character of the skin tests, corresponded to DTH.

The aim of this investigation was to study the principles governing development of this DTH in the early stages of sensitization (in the first 2 weeks), to investigate dependence of DTH on the dose of EB and the dose and type of protein antigens, and to study its nature in experiments with passive transfer of sensitivity.

EXPERIMENTAL METHOD

CBA (male or female) mice weighing 14-18 g were used in the experiments, with five to ten animals in each group. The animals were sensitized subcutaneously in the interscapular region with antigens: bovine serum albumin (BSA) from Serva (West Germany) or of USSR origin, methylated BSA (MBSA) or ovalbumin (OA), from Serva. A solution of EB (from Serva, or from Reanal, Hungary) was injected simultaneously into the same region. In the positive control group the animals were sensitized with antigen mixed with Freund's complete adjuvant, in a ratio of 1:1 with antigen solution, and with a total volume of mixture of 0.2 ml.

To assess the intensity of the developing BTH the widely tested method of injecting antigen into the animals' paws [2, 7] was used. A solution of antigen was injected into the right hind footpad in a volume of not more than 0.04 ml. The same volume of distilled water or physiological saline, in which the antigens were made up, was injected into the contralateral (control) footpad. Different sensitizing doses of antigens were tested (from 50 to 2000 μg per mouse) with different doses of EB as adjuvant (from 50 to 2000 μg per mouse) and with different times of injection of the reacting dose (from the 2nd to the 14th day after sensitization). The local inflammatory reaction was assessed 24 h later, by measuring the difference in weight of the experimental (Pe) and control (Pc) paws of each mouse, and calculating the arithmetic mean and its error (M ± m). The reaction index (RI) was determined by the formula

$$\frac{P_e - P_c}{P_c} \times 100\%.$$

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TABLE 1. Dependence of Intensity of DTH in CBA Mice on Dose of EB during Sensitization of Mice with MBSA

Group No.	Dose of EB, μg/mouse	Reaction index, % (M ± m)	p
1	2000	11.4±0.75	<0.001
2	1000	16.8±1.7	<0.001
3	500	22.1±1.0	<0.001
4	250	28.9±2.0	<0.001
5	125	20.8±1.6	<0.001
6	50	20.8±2.3	<0.001
7	Intact animals (control)	3.1±0.40	—
8	0.1 ml FCA (Difco, USA)	19.8±3.2	<0.002

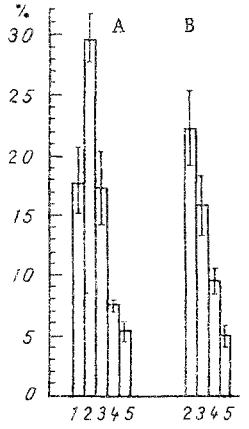

Fig. 1

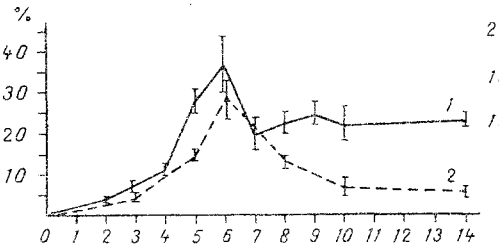

Fig. 2

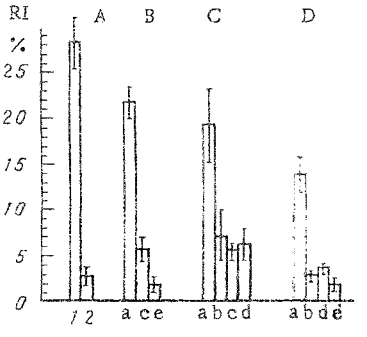

Fig. 3

Fig. 1. Intensity of DTH during sensitization of CBA mice with different doses of MBSA and with a standard dose of EB. A) EB used as adjuvant; B) Freund's adjuvant used. 1) Sensitization of mice with MBSA in dose of 50 μg, 2) 250 μg, 3) 500 μg, 4) 2000 μg, 5) intact mice. Here and in Figs. 2 and 3: ordinate, RI (in %).

Fig. 2. Time course of development of DTH in mice sensitized with mixture of EB and MBA (1) or EB with OA (2). Abscissa, time after sensitization (in days).

Fig. 3. Local transfer of DTH by spleen cells of actively sensitized mice. A) DTH reaction in actively sensitized (1) and intact (2) mice after reacting dose of MBSA; b, c, d) three experiments with transfer of DTH: a) injection of sensitized splenocytes and MBSA, b) injection of sensitized splenocytes only, c) injection of normal splenocytes and MBSA, d) injection of normal splenocytes only, e) injection of MBSA only.

The results were subjected to statistical analysis by Student's test.

In experiments with local transfer of DTH, carried out after determination of the conditions for optimal manifestation of the reaction to MBSA, spleen cells from immunized and intact mice were washed 3 times with medium 199 and injected, in a volume of not more than 0.04 ml ($2.5 \cdot 10^7$ viable cells) into intact syngeneic mice into both hind footpads, and 2 h later 25 μg of MBSA was injected into the right footpad and RI calculated after 24 h.

EXPERIMENTAL RESULTS

In the first series of experiments the effect of various sensitizing doses of antigen (MBSA: 50, 250, 500, and 2000 μg per mouse), injected with EB (250 μg per mouse) was studied on the level of the DTH reactions, when the reacting dose of 25 μg antigen per mouse was given on the 7th day. It will be clear from Fig. 1 that a dose of MBSA of 250 μg per mouse is optimal, whether EB or FCA is used. With an increase in the dose of MBSA to

2000 μg per mouse RI decreased significantly, whereas smaller doses induced a marked reaction whichever adjuvant was used.

Dependence of the level of DTH induced by a single dose of MBSA (250 μg per mouse) and by different doses of EB (from 2000 down to 50 μg per mouse) was next studied. The mice (10 animals in the group) received an injection of 25 μg of antigen on the 7th day of sensitization. The experimental results are given in Table 1. The highest value of RI was observed after doses of EB from 50 to 500 μg (highest of all from 250 μg) but some decrease in RI was observed with doses of EB from 1000 to 2000 μg. The use of FCA also produced an adjuvant effect when DTH against MBSA was studied.

Since MBSA is a fairly powerful immunogen, the adjuvant effect of EB on DTH to three types of antigen (MBSA, BSA, and OA) was compared: the last two of these are considered to be weak immunogens for CBA mice. EB was shown to stimulate the development of DTH to MBSA and OA, but not to BSA. No cross reactions of DTH were found to these three antigens. The results are evidence that EB promotes the development of DTH in CBA mice but not to all types of antigens; however, DTH does arise even to such weak antigens as OA.

To study the time course of development of DTH special experiments were undertaken on mice immunized with MBSA or OA (250 μg per mouse in each case) together with EB (250 μg per mouse), with injection of the reacting doses of the corresponding antigens (25 μg per mouse) into the paws on the second day, and subsequently until the 14th day. As Fig. 2 shows, on the 2nd-3rd day the level of DTH even to MBSA did not differ significantly from the control ($P > 0.05$). By the 5th-7th day a marked reaction to MBSA but a weaker reaction to OA were recorded. On the 10th-14th day the level of DTH reactions to MBSA remained the same as before, but to OA it fell to the control values. RI in the control animals did not exceed 5%.

To prove the cellular nature of the hypersensitivity which develops when EB is used as adjuvant, experiments were carried out with local transfer of spleen cells of actively sensitized donors into intact recipients. The results of some of the experiments are given in Fig. 3. As a first step RI was determined in mice sensitized with MBSA together with EB (250 μg of each per mouse) on the 5th-6th day after sensitization; it was 28.6 ± 2.7% compared with 2.9 ± 0.3% in the control. On the 6th-7th day splenocytes were transferred from the immune mice to syngeneic recipients, and various control tests were carried out (with and without MBSA, with immune and intact splenocytes). All the tests gave the same result: all the passively sensitized animals developed a reaction which was much stronger than that in the control mice. The possibility of transfer of hyposensitivity by means of effector cells demonstrates the cell-mediated character of this type of sensitivity.

It was thus shown by the use of the writers' modification of the method of induction of DTH that EB is a convenient and effective adjuvant for producing DTH and studying the mechanisms of its development.

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