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CORNEAL TEST AS A RELIABLE METHOD FOR DETECTION OF THE DELAYED-TYPE HYPERSENSITIVITY REACTION IN MICE

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SUMMARY Antigen solution could be injected into the cornea of sensitized mice using a fine needle and a stereoscopic dissecting microscope. The resulting corneal reaction was shown to be a reliable method in the detection and estimation of delayed-type hypersentivitity in mice that had been immunized with a water-in-oil emulsion containing an ovalbumin and a cell wall adjuvant. Unlike the delayed skin reaction in the ear lobe, this corneal reaction was not affected by a coexisting Arthus reaction.

INTRODUCTION

Cell walls isolated from a variety of bacterial species induce a cell-mediated immune response and stimulate antibody production against a protein antigen when incorporated into water-in-oil emulsion (Adam et al., 1972; Kotani et al., 1975a; Stewart-Tull et al., 1975). This immunoadjuvant activity is due to *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide or MDP), a structure common to most cell wall peptidoglycans of parastic bacteria (Ellouz et al., 1974; Kotani et al., 1975b). The immunoadjuvancy of bacterial cell walls and muramyl peptides, in induction of delayed-type hypersensitivity (DTH) to protein antigen, has been demonstrated in guinea pigs (Stewart-Tull, 1983). However, there are no reports on whether cell walls and muramyl peptides can induce DTH in mice, the laboratory animal most widely used in immunological research. This seems to be due to the lack of a reliable method for establishing DTH in the presence of a possible Arthus reaction in mice. DTH reactions in mice have been determined by measurement of a delayed

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reaction, induced by injection of a sensitizing antigen, in terms of (i) a footpad or ear lobe swelling (Crowle, 1959; Christie, 1975; Ruddle, 1978; Corsini, Bellucci and Costa, 1979), (ii) a delayed-type skin test (Dekaris and Allegretti, 1968), or (iii) the accumulation of radio-labelled cells at the site of challenge in the ear lobe (Vadas et al., 1975; Robinson and Naysmith, 1976). However, immediate-type hypersensitivity can interfere with these reactions, because a severe Arthus reaction can remain conspicuous for 24 h or more. Therefore, it is not easy by these methods to distinguish an Arthus reaction from a delayed-type reaction, either by gross inspection or by a more quantitative estimation.

On the other hand, the corneal reaction is not influenced by an overriding Arthus reaction because there are few functional blood vessels in the cornea except those in the limbal circulation. Therefore, the corneal test has been found to be a reliable assay method for DTH in guinea pigs, rats and rabbits (Raffel et al., 1949; White, Coons and Connolly, 1955; Elliot, Flax and Leibowitz, 1966; Chandler, Eugene and Russell, 1973; Stewart-Tull et al., 1975; Friedlaender and Dvorak, 1977; Tanaka et al., 1977). However, so far there have been no reports on the use of the corneal test in mice, presumably due to the technical difficulty of inoculation. In this work, we found that the corneal test was a reliable method for detection and estimation of DTH in mice that had been sensitized with ovalbumin as a test antigen and *Nocardia canicruria* or *Nocardia corynebacteriodes* cell walls as an adjuvant in a water-in-oil emulsion.

MATERIALS AND METHODS

1. *Immunization.*

Groups of eight 5-week-old male mice [inbred C3H/He, BALB/c or a closed colony ICR from Charles River Japan (Kanagawa, Japan)] were given an injection of 0.05 ml of a water-in-oil emulsion in the left hind-footpad. The emulsion was prepared by sonication of a mixture (1 : 1, v/v) of Bacto Incomplete Freund's Adjuvant (IFA; Difco Laboratories, Detroit, Mich., USA.) and 0.01 *M* phosphate-buffered saline (pH 7.0) containing 4.0 mg ovalbumin (Grade V; Sigma Chemical Co., St. Louis, Mo., USA.) and 2.0 mg of either *N. canicruria* (ATCC 17896) or *N. corynebacteriodes* (ATCC 14898) cell walls. A dose of 100 μg of antigen and 50 μg of adjuvant was injected. The corneal test was done in one eye 21 days later. Animals received a booster injection 3 days later in the right hind-footpad of the same water-in-oil emulsion as used for the first immunization. Seven days after the booster injection, another corneal test was done in the other eye. Control mice were immunized with a water-in-oil emulsion containing ovalbumin but not cell wall adjuvant.

Cell wall preparations were prepared as described previously (Takada et al., 1979), and kindly supplied by Drs. Kanae Yokogawa and Shigeo Kawata (Dainippon Pharmaceutical Co., Osaka, Japan).

2. *Corneal Test.*

A mouse was anesthetized with ether and placed under a stereoscopic dissecting microscope (magnifying power ×6.3, Model JMTr.,; Olympus Co., Tokyo, Japan). Then ovalbumin solution (20 mg/ml of physiological saline solution) sterilized by filtration through a Millipore filter (pore size 0.45 μm; Nihon Millipore Ltd., Tokyo) was injected into the cornea using a syringe with a fine needle (specially made with an outer diameter of 0.28 mm, and 16 mm in length with a short bevel cut to an angle of 45°; Misawa Kogyo Co., Tokyo). The needle was inserted tangentially to the curvature of the eye into the center of the cornea. The amount of antigen injected was adjusted to produce a discrete opaque disc of about 1.5 mm diameter in the cornea (Fig. 1). The volume of antigen solution was approximately 0.5 μl containing 10 μg of ovalbumin. The eyes of test mice were examined 24 and 48 h later by naked eye under appropriate illumination. The intensity of the reaction provoked by DTH was graded as follows: (0), clear transparent cornea, (1), slightly cloudy with a distinct pupil, (2), cloudy and pupil hidden, (3), completely opaque with a rough surface. In general, 0 or 1 were scored as negative and 2 and 3 as positive. The mean scores of the corneal response in each group were recorded.

3. *Ear-Lobe Hypersensitivity Reaction.*

Ten days after the booster injection and three

FIGURE 1. Injection of antigen solution (approximately 0.5 μl of 20 mg/ml ovalbumin solution) into a mouse cornea. The bevelled tip of the needle is in contact with the corneal surface.

days after the second corneal test, ovalbumin solution (1.0 mg/ml of physiological saline) was injected intradermally into the ear lobe to form a bulla of approximately 2 mm in diameter (about 10 μl antigen solution containing 5 μg of ovalbumin was injected). At 2 h intervals for 12 h, and then every 24 h for four days after the injection, the thickness of the lobe at the injection site was measured with a dial gauge (Model SM-528; Teclock Corp., Tokyo).

4. *Estimation of Antibody Level.*

After the corneal test, mice were bled from the retro-orbital plexus, and the anti-ovalbumin level of their serum was determined by a reversed single radial immunodiffusion technique. Triplicate 10 μl serum samples from individual mice were put into wells of an agarose plate containing ovalbumin (3.0 μg ovalbumin/ml of 1.0% agarose gel). These plates were incubated for 24 h at room temperature in a moist chamber, and then the diameters of the precipitin zones were measured. Values were standardized by comparison with that for pooled reference serum of the ICR mice immunized with ovalbumin plus adjuvant, which contained 350 μg antibody nitrogen/ml. The lower limit of accurate determination was about 25 μg antibody nitrogen/ml of test serum.

5. *Histology of Cornea*

Cellular infiltration into the cornea was examined histologically in animals killed at 6, 24, 48, and 72 h after the corneal test. The eyes were removed and fixed in 10% formalin-saline solution. The fixed eyes were embedded in paraffin wax and section of 5 μm thickness were cut and stained with hematoxylin and eosin.

RESULTS

Figure 2 shows positive and negative corneal reactions 48 h after injection of the ovalbumin solution into the cornea of albino ICR mice with red eyes (A) and dark-brown haired C3H/He mice with black eyes (B) which had been sensitized with ovalbumin and *N. canicruria* cell walls given as water-in-oil emulsion. The opaque disc formed by the initial injection of ovalbumin solution into the cornea disappeared within 1 h. But after 12 h, the cornea of the sensitized mice became cloudy to an extent depending on the level of DTH induced, and the cloudiness caused by positive delayed hypersensitivity to ovalbumin per-

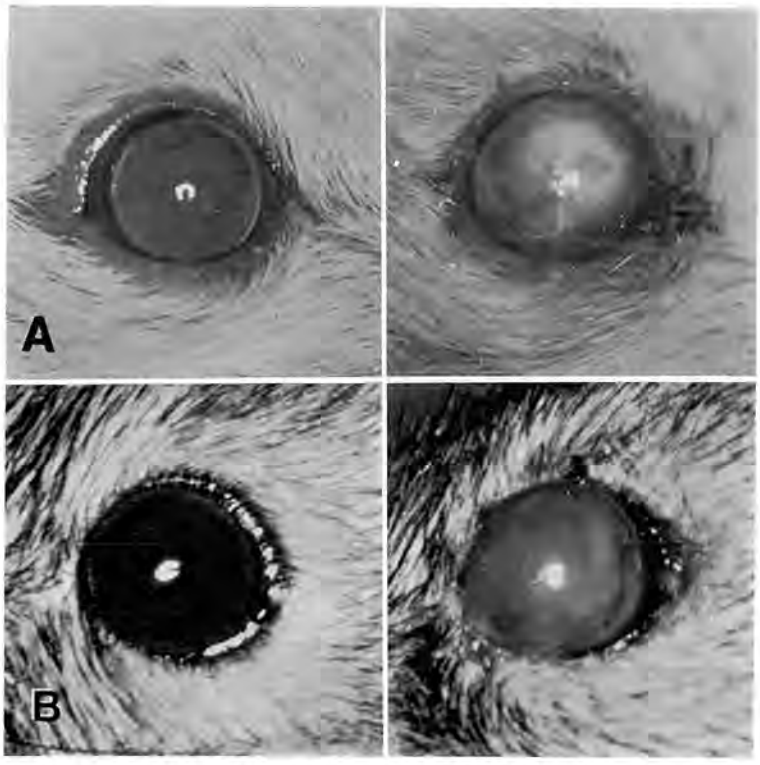

FIGURE 2. Corneal responses of mice (48-h after the antigen injection). The responses in A) ICR mice and B) C3H/He mice are shown. The left side shows a negative corneal reaction, and the right a positive one.

sisted for 48 h, at least.

Figure 3 shows the histological appearance of an eye giving a positive corneal reaction. At an early stage, cellular infiltration of a number of neutrophils and a few monocytes / macrophages is recognizable, predominantly in the corneo-scleral limbus area. At a later stage, 48 h after the injection, the infiltration spread to the central area of the corneal tissue. These histological findings were essentially the same as those observed in guinea pigs (Long and Holley, 1933; Friedlaender and Dvorak, 1977), but in mice, polymorphonuclear cells were also observed among the infiltrating cells, whereas in guinea pigs these cells were exclusively monocytes/macrophages. Similar findings in a DTH dermal reaction of sensitized mice against protein or polysaccharide antigen were reported by Crowle (1975).

The time course of the ear lobe hypersensitivity reaction in mice sensitized with ovalbumin and *N. corynebacteriodes* cell walls is shown in Fig. 4. The reaction was biphasic with a trough at 12 h after injection of provocative antigen into the ear lobe. The early and late phase reactions seemed to be mainly due to the Arthus reaction and DTH, respectively. However, these two reactions showed considerable overlap making it difficult to distinguish the two reactions clearly and to determine the extent of the induced DTH quantitatively. The immunological specificity of the ear lobe reaction was confirmed by the finding that after injection of bovine serum albumin (crystallized and lyophilized; Sigma) solution (1.0 mg/ml of physiological saline), which is heterologous to the antigen used for sensitization, no significant swelling occurred

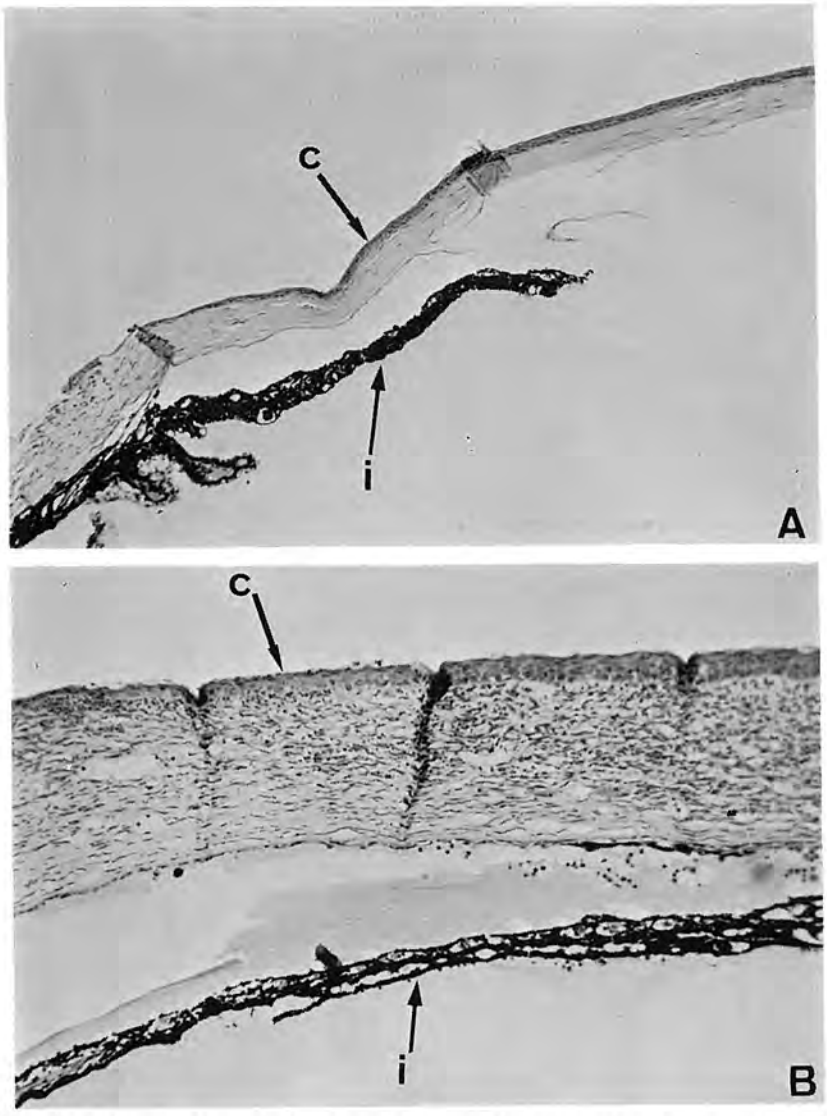

FIGURE 3. Histological comparison of A) a normal, or negative eye reaction of a mouse sensitized with the antigen alone with B) a positive corneal reaction in a C3H/He mouse sensitized with antigen and *N. corynebacteriodes* cell walls. Eyes were removed 48 h after antigen injection. Arrow " c " indicates the cornea (thickness: A, 50–70 μm, B, 150–200 μm) and " i " indicates the iris. Hematoxylin and eosin stain.

during the time course of either phase of the ear lobe reaction.

The corneal reactions, together with ear lobe reactions and circulating antibody levels in ICR, BALB/c and C3H/He mice that had been sensitized with ovalbumin and *N. canicruria* cell walls in a water-in-oil emulsion, are summarized in Fig. 5. All the test mice of these three strains exhibited a strong positive corneal reaction, while no control mice immunized

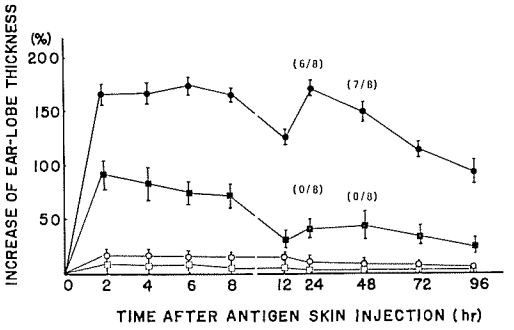

FIGURE 4. Time course of the ear lobe reaction in BALB/c mice sensitized with ovalbumin with or without *N. corynebacteriodes* cell walls in a water-in-oil emulsion. Results show ear lobe reactions after injection of antigen solution (approximately 5 μl of 1 mg/ml ovalbumin solution) in mice immunized with ovalbumin with (●) or without (■) the cell wall adjuvant, and after injection of bovine serum albumin solution in mice immunized with an ovalbumin with (○) or without (□) the cell wall adjuvant. Values in brackets indicate the ratio of mice giving positive corneal reaction to total mice examined at each time.

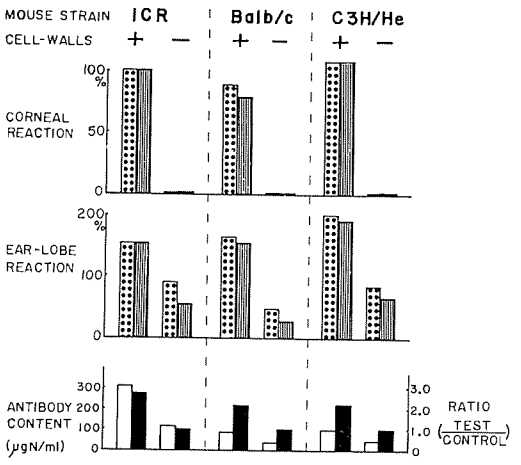

FIGURE 5. Responsiveness of three strains of mice to the immunoadjuvant activity of *N. canicruria* cell walls. Mice were sensitized by an injection into the footpad of 100 μg of ovalbumin with (+) or without (−) the cell walls (50 μg) in a water-in-oil emulsion. The corneal test was done by injection of antigen solution into one eye (approximately 0.5 μl containing 10 μg of ovalbumin) three weeks after the first immunization and was repeated in the other eye one week after the booster injection. The second corneal reaction is shown as the percentage of animals showing a positive reaction [24 h response (▨), 48 h response (▥)]. The ear lobe reaction elicited by injection of antigen solution (approximately 5 μl containining 5 μg of ovalbumin) is shown as the percentage increase in thickness of the ear after the injection [24 h response (▨), 48 h response (▥)]. The antibody content is presented as μg antibody nitrogen/ml of serum (□) or as the ratio of the antibody content in the sera of the test group to that of the control group (■). Note the clear-cut distinction of positive and negative corneal responses in animals sensitized with antigen with or without *N. canicruria* cell walls as an adjuvant in water-in-oil emulsion, respectively, but the less clear distinction between these groups in the ear lobe reaction. In addition, the ear lobe reaction is related to the level of antibody indicating possible interference from an Arthus reaction.

with ovalbumin alone in a water-in-oil emulsion showed an appreciable corneal reaction. The differences between the corneal reactions in animals sensitized with the antigen in the presence and absence of *N. canicruria* cell walls were clear-cut, that is essentially positive or negative. In the ear lobe hypersensitivity reaction, there were definite differences between test and control animals in the percentage increase in the thickness at the injection site of the provocative antigen, but the differences were not clear-cut. The circulating serum anti-ovalbumin antibody levels were higher in mice immunized with a water-in-oil emulsion containing ovalbumin as an antigen and the cell walls as an adjuvant than in mice treated with a water-in-oil emulsion containing antigen alone.

DISCUSSION

The present results show that the corneal delayed-type hypersensitivity reaction in mice is a useful and reliable assay method for evaluation of DTH in mice, as it has been found to

be in guinea pigs. The advantage of the corneal test is that the reaction is scarcely influenced by a coexistent Arthus reaction because of the functional avascularity of the corneal tissue. This was verified by histological examination of corneal tissue from mouse eyes showing a positive reaction. Histological examination showed that the corneal opacity recognized by naked eye was due to the combined effects of edema, inflammatory cell infiltration, deposition of fibrin and dearrangement of inherent corneal cells, as observed previously in guinea pigs (Salvin and Gregg, 1961; Friedlaender, 1979).

The technical difficulties of the injection due to small size of the mouse eye may be overcome by the use of a fine needle and stereoscopic dissecting microscope. Conventional methods for detecting DTH in mice (Crowle, 1975), such as the footpad swelling reaction or ear lobe thickening reaction, are technically easy, but have the serious disadvantage that an Arthus reaction interferes with measurements of the DTH reaction, particularly after repeated immunizations, which result in elevation of serum antibody. Another difficulty is inaccurate measurement of footpad volume or ear lobe thickness.

In practice, it is unnecessary to fix the amount of test antigen solution to be injected into the cornea precisely, because the antigen concentration is high. Moreover, this test can be made on various strains of test animals, including both albino mice with red eyes and coloured mice with black eyes. Thus, the potencies of various test adjuvants to induce DTH in mice may be compared effectively under essentially standard conditions.

The ear lobe reaction was primarily devised to determine the DTH dependent on T cell activation at an early stage of the immune response (Van Loveren, Meade and Askenase, 1983; Sewell, Munoz and Vadas, 1983). We have also observed that the ear lobe reaction was weakly positive in mice that had received one immunization with ovalbumin plus cell walls, while the corneal test was negative at this stage (data not shown). But we do not know whether this means that the sensitivity of the corneal test is less than that of ear lobe reaction.

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