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Dimethyl dioctadecyl ammonium bromide as adjuvant for delayed hypersensitivity in mice

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Summary. Immunization of mice with antigen mixed with the cationic surface active lipid dimethyl dioctadecyl ammonium bromide (DDA) produced delayed type hypersensitivity (DH), measured as a footpad swelling. The DH to sheep red blood cells or dinitrophenyl conjugated with bovine serum albumin (DNP_{28}-BSA) in DDA exceeded the response of the same antigens in Freund's Complete Adjuvant (FCA) significantly. Treatment of mice with CY 8 hr prior to the injection of antigen in FCA or DDA resulted in delay of the onset of footpad swelling past day 5 and in elimination of the differences in the response due to the adjuvants.

Immunization with carrier or hapten-carrier complexes with different epitope density in DDA and elicitation with the homologous and heterologous antigens revealed that the DH was DNP-specific.

In vivo priming with DNP_{28}-BSA in DDA and *in vitro* stimulation with the same antigen resulted in peak responses which were twice as high and were reached almost twice as fast as the earlier found response following immunization in FCA. The advantages of DDA as adjuvant over covalently linked fatty acid chains and over FCA are discussed.

INTRODUCTION

Numerous experimental systems have been devised for enhancing the induction of delayed type hypersensitivity (DH) to protein antigens by conjugating them with haptens or other materials (Turk, 1967; Gell & Benacerraf, 1961; Parish, 1971). In addition many of these protein conjugates required FCA for the production of a lasting DH. Coon & Hunter (1973, 1975) used BSA heavily conjugated with fatty acids to induce in guinea pigs strong sustained DH to BSA without detectable antibody formation. The fatty acids had to be covalently linked to BSA; the mere mixing of the two did not detectably change the immunogenic properties of BSA.

Double conjugation of BSA with fatty acids and DNP produced DH to the hapten (Dailey & Hunter, 1974). The introduction of fatty acids in a protein has the disadvantage that the linkage of both fatty acid residues and DNP groups are attached mainly to the amino-lysine groups of the protein. This excludes the use of hapten carrier complexes with a high epitope density. Furthermore the conjugation technique is not readily available in most laboratories. For these reasons we extended the experiments with a cationic surface active lipid, dimethyl dioctadecyl ammonium bromide (DDA) of Dailey & Hunter (1974). Mixing with DDA introduced into the antigen lipid groups which are attached by electrostatic bonds. We studied the use of DDA in mice as an adjuvant for a particulate antigen (sheep red blood cells, SRBC) and for DNP-BSA complexes with different epitope densities.

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MATERIALS AND METHODS

Animals and immunization

Inbred female BALB/c mice were bred and maintained in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. For each experiment groups of six to eight mice of an age of about 12 weeks were used. Different amounts of antigen dissolved in 0·1 ml saline, and unless otherwise stated, emulsified with a suspension of 100 μg dimethyl dioctadecyl ammonium bromide (Eastman Kodak, New York) in 0·1 ml saline or with 0·1 ml Freund's complete adjuvant (FCA) containing killed Mycobacterium $H_{37}Ra$ (Difco) were injected intracutaneously (i.c.) in the abdomen divided over four sites.

Antigens and mitogens

The 2,4-dinitrophenyl group (DNP) was used as hapten. DNP-protein coupling was carried out and analysed according to the method of Eisen, Carsten & Belman (1954) with 2,4 dinitrobenzene sulphonic acid sodium salt (Eastman Kodak, New York), and yielded products with the following ratios: DNP_3-BSA, DNP_{16}-BSA, and DNP_{28}-BSA. DNP_{50}-BSA was prepared according to Klaus & Cross (1974) using 2,4-dinitrofluorobenzene. Sheep red blood cells (SRBC) in Alsever's old solution were obtained from the National Institute of Public Health, Bilthoven, The Netherlands.

Assay for delayed hypersensitivity (DH)

The DH reactions were determined by measuring the increase in footpad thickness (footpad swell test) as described by Kerckhaert, Van den Berg & Willers (1974) using a semi-electronic paw meter (Van Dijk, Versteeg & Hennink, 1976). The eliciting dose was given by an injection into the left footpad of 25 μg DNP-BSA or 4×10^6 SRBC suspended in saline to a total volume of 0·05 ml. A footpad swelling of 0·25 mm is regarded as positive. The results were expressed as the increment of the footpad thickness.

Cyclophosphamide (CY) treatment

CY was obtained from Koch-Light Laboratories (Colnbrook, Bucks, England). The mice received an intraperitoneal (i.p.) injection of 300 mg CY/kg in 0·5 ml saline, 8 hr before immunization.

Specific stimulation

Stimulation (total incubation time 64 hr) and the [^{3}H]-thymidine incorporation assay were done as described previously (Snippe, Nab & Van Eyk, 1974). The ratio of incorporation (R value) was calculated by dividing the number of disintegrations per minute (dpm) for the stimulated culture by the number of dpm for the unstimulated culture using a programmable calculation apparatus (Alphatronic, Diehl, FRG).

Statistical analysis

Results are expressed as the arithmetic mean of n values $\pm$ the standard error of the mean (S.E.M.). Student's t-test was performed to analyse the statistical significance of the results. P values $> 0{\cdot}05$ are considered non-significant (N.S.).

RESULTS

Effect of DDA on cell numbers in the lymphoid organs

Groups of five mice were immunized with 50 μg of DNP_{28}-BSA alone or emulsified in 10 or 100 μg of DDA. On varying days after immunization the numbers of cells in spleen, inguinal lymph nodes, bone marrow and thymus were determined by haemocytometer count (Veldkamp, Gaag & Willers, 1973). No effect of DDA on the spleen cell numbers was found (Fig. 1). The cell numbers in the lymph nodes were reduced to 50% on day 9 and returned to normal values by day 20. The effects on bone marrow and thymus were almost identical, after an initial reduction of cell numbers of 25–50%, normal values were reached again by day 16.

Time course of footpad swelling after eliciting injection

Groups of ten mice were immunized with 4×10^7 SRBC in DDA or FCA and challenged with SRBC in saline. The footpad swelling was measured at several intervals after the eliciting injection. From Fig. 2 it is clear that the curves for footpad swelling following immunization with antigens in DDA and FCA showed a similar pattern with a peak at 24 hr. The curve obtained with DDA was however significantly higher than the FCA curve between 10 and 24 hr after challenge ($P < 0{\cdot}005$).

Effect of CY treatment on DH

Previously Kerckhaert, Berg & Willers (1974)

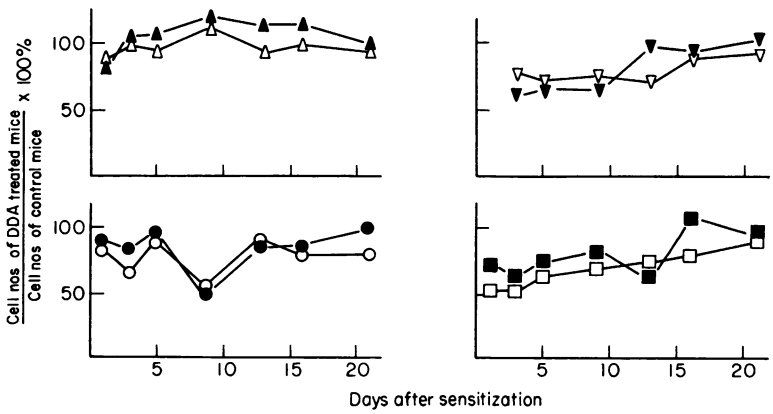

Figure 1. Effect of DDA on cell numbers in lymphoid organs. Groups of five mice received 50 μg DNP_{28}-BSA in DDA or in saline (controls). On varying days following the immunization the cell numbers in spleen (△), bone marrow (▽), inguinal lymph modes (○) and thymus (□) were determined. The relative value of cell numbers following treatment with 10 μg (open signs) or 100 μg of DDA (filled signs) is given.

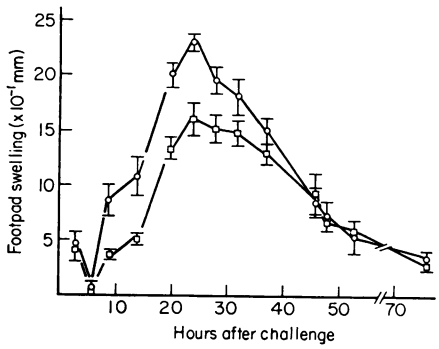

Figure 2. Time course of the footpad swelling. Groups of mice were immunized with 4×10^7 SRBC in FCA (□) or DDA (○) and challenged 5 days later with 4×10^6 SRBC. The footpad swelling was measured at varying hours after the challenge. Vertical bars indicate standard errors of the mean. *P* values between 10 and 30 hr of DDA versus FCA treated groups: $P < 0{\cdot}005$.

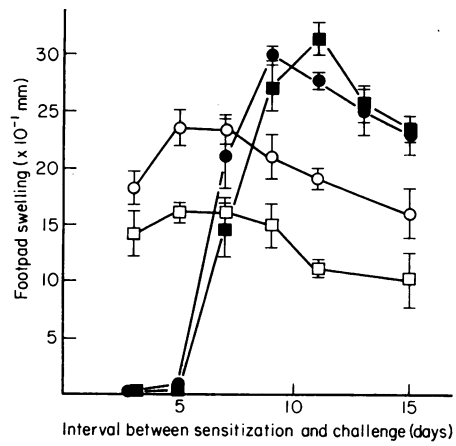

Figure 3. The effect of CY treatment on DH to SRBC. Groups of six mice were immunized i.c. with 4×10^7 SRBC in FCA (□) or DDA (○) 8 h after i.p. injection of saline (open signs) or CY (filled signs). At varying days after immunization 4×10^6 SRBC were injected into the left footpad.

demonstrated that a single injection of CY preceding i.c. injection of SRBC in FCA resulted in a delayed but appreciably enhanced DH. In the following experiment the effect of substitution of DDA for FCA was studied. Groups of five mice were treated with CY or saline and 8 hr later injected with 4×10^7 SRBC in FCA or DDA. After different intervals groups of mice were tested with SRBC. The use of FCA in non-CY treated mice resulted in a high level of footpad swelling between days 3 and 9 (Fig. 3). The footpad swelling following DDA application resulted in significantly enhanced footpad swellings for the whole observation period. CY treatment delayed the onset of the footpad swelling past day 5. However the differences in footpad swelling between DDA and FCA injected animals were almost eliminated after CY treatment.

In order to study the effects of DDA in more detail further experiments were performed with hapten-carrier complexes. Groups of mice were immunized with 50 μg of DNP_{28}-BSA in FCA or DDA,

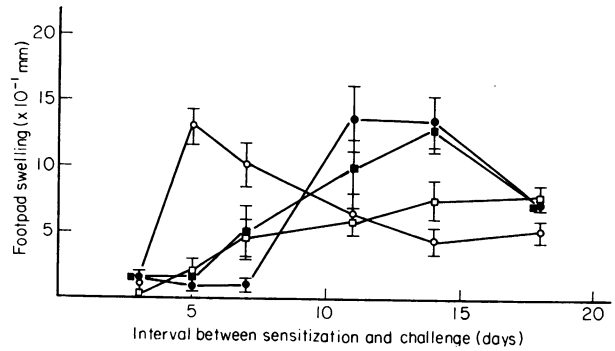

Figure 4. The effect of CY treatment on DH to hapten-carrier complexes. Groups of six mice were immunized i.c. with 50 μg DNP_{28}-BSA in FCA (□) or DDA (○) 8 h after i.p. injection of saline (open signs) or CY (filled signs). At varying days after immunization 25 μg DNP_{28}-BSA was injected into the left footpad.

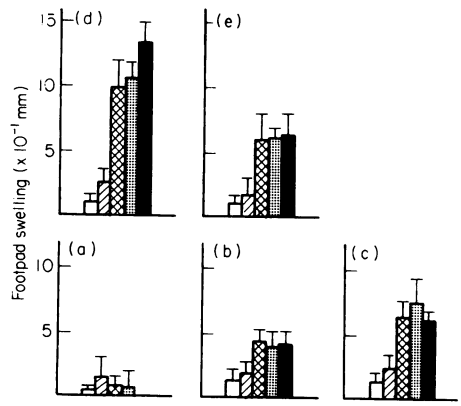

Figure 5. Homologous and heterologous DH reactions with carrier and hapten-carrier complexes. Groups of thirty mice were immunized with 50 μg of BSA (a), DNP_3-BSA (b), DNP_{16}-BSA (c), DNP_{28}-BSA (d) or DNP_{50}-BSA (e). At day 5 of each group six mice were challenged in the left footpad with 25 of μg BSA (open column), DNP_3-BSA (hatched column), DNP_{16}-BSA (cross-hatched column), DNP_{28}-BSA (dotted column) or DNP_{50}-BSA (black column).

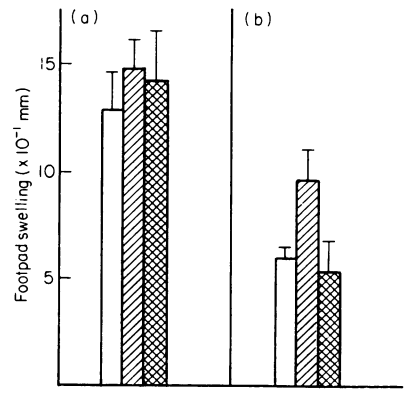

Figure 6. DH on highly haptenated DNP-BSA. Groups of mice were immunized with 10 (a) or 100 μg DNP_{50}-BSA in DDA (b) and challenged at day 5 with 25 μg DNP_{16}-BSA (open column), DNP_{28}-BSA (hatched column) or DNP_{50}-BSA (cross-hatched column).

followed by 25 μg of DNP_{28}-BSA as eliciting dose. From Fig. 4 it is clear that the DH following FCA became positive after day 5 and increased steadily. Mice receiving DDA showed a peak DH on day 5, which far exceeded the maximal values of the FCA-injected mice. CY treatment delayed the onset of the DH of DDA treated mice considerably. The peak response which was the same for both FCA and DDA-injected mice did not exceed that of the non-CY treated DDA injected animals. The net result of CY treatment and immunization with antigen in DDA was an extended peak response.

Homologous and heterologous reactions with carrier and hapten-carrier complexes in the DH

Snippe, Willems, Graven & Kamp (1975) showed that priming with BSA in FCA induced strong DH against BSA but DH of decreasing strength against complexes with increasing DNP/BSA ratio. Priming with DNP/BSA complexes never resulted in DH against BSA or DNP_3-BSA. Injections of DNP_{16}-BSA and DNP_{28}-BSA induced a DH which increased with the hapten/carrier ratio of the eliciting

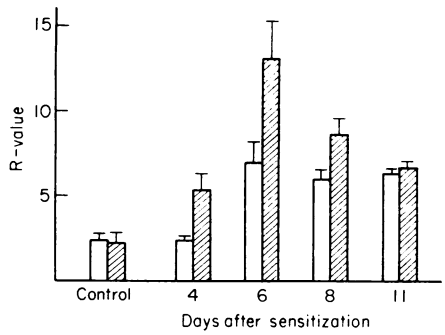

Figure 7. Stimulation of primed mouse spleen cells by DNP-carrier complex. Groups of mice were immunized with 0 (control), 10 (open column) or 100 μg (hatched column) DNP_{28}-BSA in DDA. At different days after sensitization pooled spleen cells were cultured in triplicate *in vitro* with 100 μg DNP_{28}-BSA for 64 h and the last 24 h pulsed with 1 μCi of [^{3}H]-thymidine. Results are given as *R*-values.

antigen. This experiment was repeated with DDA as adjuvant. Groups of mice were primed with 50 μg of BSA or DNP-BSA complexes, followed 6 days later by a challenge with BSA or DNP-BSA complex. No positive DH could be measured when mice were primed with BSA (Fig. 5a). When DNP-BSA complexes were used for priming, no positive reaction was obtained when BSA or DNP_3-BSA were used as eliciting antigen (Fig. 5b–e). This was not due to the dose used for challenge as either increase or decrease of the dose did not alter the result. Clear cut reactions were obtained when DNP_{16}-BSA, DNP_{28}-BSA and DNP_{50}-BSA were used for elicitation. Unexpectedly DNP_{28}-BSA seemed a better immunizing antigen than DNP_{50}-BSA. To test if this effect was caused by the immunizing dose, groups of animals were immunized with 10 or 100 μg of DNP_{50}-BSA in DDA (Fig. 6). The animals received an eliciting injection with 25 μg of DNP_{16}-BSA, DNP_{28}-BSA or DNP_{50}-BSA. It is clear that following immunization with 10 μg of DNP_{50}-BSA DH are obtained which at least equal those obtained with DNP_{28}-BSA (Fig. 6).

Specific stimulation of spleen cells *in vitro* after *in vivo* immunization with antigen in DDA

In previous experiments Snippe, Nab & Van Eyk (1974) found 11 days after *in vivo* priming with antigen in FCA to be optimal for *in vitro* stimulation of spleen cells with the same antigen. In the next experiment mice were primed with 10 or 100 μg of DNP_{28}-BSA in DDA and after different intervals the spleens of these mice were used for *in vitro* stimulation with 100 μg of DNP_{28}-BSA.

From Fig. 7 it is clear that the highest *R* values were obtained 6 days after immunization with 100 μg of antigen. *In vitro* stimulation with 10 μg of DNP_{28}-BSA resulted in far lower responses (not shown).

DISCUSSION

The difference in effect on cell numbers in lymph nodes and spleen following the application of DDA can be explained by the intracutaneous route of injection which primarily affects the lymph nodes. The reason for the reduction in cell numbers in lymph nodes and in bone marrow and thymus however cannot be given. Further experiments determining the target cells in the lymph nodes will be necessary. Our results show that DDA could be used as adjuvant for SRBC. The footpad swelling following the use of DDA exceeded significantly the values obtained with FCA over the whole observation period. The delay in the onset of DH and the enhancement of the peak response following CY treatment shortly before immunization with antigen in FCA (Kerckhaert *et al.*, 1974) was also found when the immunizing antigen was given in DDA instead of FCA (Fig. 3). With DNP_{28}-BSA in DDA the peak due to CY treatment did not exceed the early value obtained without CY (Fig. 4). With both antigens however CY treatment eliminated the differences in response following DDA or FCA application.

In earlier experiments the role of hapten and carrier in DH were studied using FCA as adjuvant (Snippe *et al.*, 1975). Priming with BSA or DNP_3-BSA induced a strong DH to the carrier but decreasing responses to complexes with increasing numbers of hapten groups. This suggested a DH to carrier determinants. On the other hand after priming with complexes, the DH increased with the hapten/carrier ratio. The hapten/carrier ratio of the complex used for elicitation was also important. The response increased from none for DNP_3-BSA to a high response for DNP_{28}-BSA.

In these experiments the DH was obviously mainly directed against the DNP groups and not against the carrier. Extending these experiments with DDA as adjuvant and including the highly haptenated DNP_{50}-BSA revealed that no DH could be measured

when mice were primed with BSA or when BSA or DNP_3-BSA were used as eliciting antigen (Fig. 5). Even the reaction of DNP_3-BSA in the homologous system was negative. Positive reactions were obtained with intermediate or highly haptenated complexes as eliciting antigen. The seemingly weaker reactivity of DNP_{50}-BSA was probably caused by the high numbers of DNP groups in the immunizing dose. Reducing this dose resulted in a response which was at least equal to that to DNP_{28}-BSA (Fig. 6). Comparison of these results and those obtained with FCA (Snippe *et al.*, 1975) suggests that immunization of mice with DNP-BSA complexes in DDA produced exclusively DNP specific DH, whereas the use of FCA permitted the production of BSA or DNP specific DH depending on the antigen used for immunization. Our results with a great range of DNP-BSA complexes in mice confirm and extend those of Daily & Hunter (1974) with DNP_{16}-BSA in guinea-pigs.

Priming of mice with DNP_{28}-BSA in FCA, followed 11 days later by *in vitro* stimulation with the same antigen resulted in *R* values of about 5 (Snippe *et al.*, 1974). The use of DDA as adjuvant resulted in a peak response which appeared earlier (day 6) and which was twice as high as in the FCA experiment. This is in agreement with the earlier appearance of peak DH following immunization in DDA as compared with FCA. From our results it is clear that DDA is an adjuvant which favours a strong DH preferentially to haptenic groups. In none of our experiments could antibody responses be measured. Further advantages of DDA over FCA are the absence of immunogenic components in DDA in contrast with the presence of immunogenic components of Mycobacteria in FCA and the lack of severe local ulceration, which always occurs at the injection site when FCA is used.

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