Immunology 1989 **67** 365–369

Flare-up reaction on murine contact hypersensitivity

I. DESCRIPTION OF AN EXPERIMENTAL MODEL: RECHALLENGE SYSTEM

N. YAMASHITA, M. NATSUAKI & S. SAGAMI *Department of Dermatology, Hyogo College of Medicine, Mukogawa-cho, Nishinomiya, Hyogo, Japan*

Accepted for publication 3 March 1989

SUMMARY

In this experiment, a 'rechallenge system' was established in BALB/c mice to study a local immunological reaction of contact hypersensitivity (CH). Briefly, mice were sensitized by a single painting with 25 μl of 0·5% dinitrofluorobenzene (DNFB) on the shaved back skin on Day 0. On Day 5, they were challenged with 20 μl of 0·2% DNFB on each left ear, and on Day 33 challenged again with either painting 20 μl of 0·2% DNFB on their both ears or intravenous administration of 15 mg of dinitrobenzene sulphonic acid (DNBS). As the result, marked ear swelling was observed by the second challenge only on the first challenged site and these responses were clarified to be antigen specific. In *in vitro* experiments, it was shown that only the cells from the regional lymph node of the skin, which were previously elicited on challenge, were enhanced to proliferate by DNBS, which was added into the culture medium. These results suggest that there is a local immunological mechanism to respond to the specific antigen only on the site in which CH reaction has been elicited previously. The results from this 'rechallenge system' may help to explain some pathological mechanisms of such chronic diseases as fixed drug eruption or chronic contact dermatitis, which recur easily in skin lesions involved previously.

INTRODUCTION

It has been experienced clinically that chronic contact dermatitis recurs easily in the same lesion as that was involved previously. This empirical fact suggests that the region of skin where an allergic inflammation has ceased once before has retained a certain hypersensitivity for the local immunological reaction, aside from some possibilities that the limited area of skin could probably be exposed to the same antigens. In order to elucidate the mechanism of recurrence of eruptions in the same location, we set up an original experimental system named the 'rechallenge system' using an experimental contact hypersensitivity (CH) model in mice.

In this 'rechallenge system', mice were sensitized by a single painting with an antigen on the shaved back skin on Day 0. On Day 5, they were challenged with the antigen on the left ear as the first challenge and ear swelling was measured to confirm an induction of sensitization. On Day 33, they were challenged again with the antigen on both ears as the rechallenge. We examined the time–course of the reaction after the rechallenge and whether the reaction was antigen specific or not.

Correspondence: Dr M. Natsuaki, Dept. of Dermatology, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663, Japan.

Next, in this 'rechallenge system' the antigen was administered systemically and the reaction in the ears was studied. So mice were sensitized and challenged first only on the left ear, and then second, then the antigen was intravenously administered 4 weeks later. The swelling of both ears after this treatment was observed.

Furthermore, as an *in vitro* experiment, 4 weeks after the first challenge lymphocytes were collected from both cervical, axillary and inguinal lymph nodes and incubated, and their proliferative reaction to the specific antigen was also studied.

It has been reported previously that inflammatory reactions occur again at the same area of skin that has shown a hypersensitivity reaction previously when the antigen is administered again either locally or systemically, in an experimental model of delayed-type hypersensitivity (DTH) reaction using guinea-pigs and mice (Klasen *et al.*, 1987; Van de Putte *et al.*, 1983; Lens *et al.*, 1984; Dahlback & Moller, 1981; Nakagawa *et al.*, 1978; Uesugi, Semma & Higuchi, 1985). It was suggested that such reactions could be caused by a mechanism of local immunological memory (Scheper *et al.*, 1983). However, in an analysis on this kind of immunological memory, there have been few studies using an experimental CH model in mice.

Our 'rechallenge system' seems to contribute some knowledge for elucidating the pathogenetic mechanism of fixed drug

eruption, which can recur in a limited area of skin, as well as for unveiling the mechanism of recurrence of chronic contact dermatitis in a previous lesion.

MATERIALS AND METHODS

Animals

BALB/c AnNCrj female mice were obtained from Charles River, Kanagawa. All mice were used at 6–8 weeks of age. Six mice were used in each experimental group. All experiments were repeated at least twice and similar results were obtained.

Sensitization

Mice were sensitized through a shaved back skin site on Day 0 by the topical application of either 25 μl of 0·5% 2,4-dinitro-1-fluorobenzene (DNFB) (Wako Pure Chemical, Osaka) or 50 μl of 7% picryl chloride (PCl) (Tokyo Kaseikogyo, Tokyo) in a vehicle consisting of a 4:1 acetone/olive oil solution (A/O).

Elicitation of contact hypersensitivity response

On Day 5, the mice were challenged by the topical application of either 20 μl of 0·2% DNFB or 20 μl of 0·5% PCl onto the left ear as the first challenge. A constant area of the ears was measured immediately before the challenge and 24 hr after with a dial thickness gauge (Peacock, Ozaki Seisakusho). Ear swelling was expressed as the difference in ear thickness before and after the challenge in units of 10^{-2}/mm $\pm$ SEM. On Day 33, the mice were challenged again by the topical application of either 20 μl of 0·2% DNFB or 20 μl of 0·5% PCl or 20 μl of a 4:1 acetone/olive oil solution as the second challenge (rechallenge). In another group, on Day 33, the mice were intravenously injected with either 15 mg of 2,4-dinitrobenzene sulphonic acid sodium salt (DNBS) (Tokyo Kaseikogyo, Tokyo) as the second challenge or 0·5 ml of saline as the control. Then each ear swelling was measured 2, 6, 12, 24, 48 and 72 hr after the rechallenge of local and intravenous administration.

In vitro *antigen stimulation*

As described above, mice were sensitized with DNFB on Day 0 and challenged with DNFB on Day 5. Then on Day 33, left cervical, right cervical, axillary and inguinal lymph nodes were removed and gently dissociated into single cell suspension in Hanks' solution. The cells were washed twice by centrifugation and suspended at 1×10^7 cells/ml in RPMI-1640 media (Osaka University, Biken) supplemented with 5% heat-inactivated horse serum (M.A. Bioproducts, Walkersville, MD). 5×10^5 viable cells were cultured in flat-bottomed, 96-well culture plates (Falcon 3072; Becton-Dickinson, Oxnard, CA)) containing 100 μl of RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 10 mM of HEPES and 5% heat-inactivated horse serum. Cultures were carried out for 96 hr at 37° in a humidified atmosphere of 5% CO_2 in the culture medium with or without DNBS. The cultures were pulsed with 0·5 μCi of [^{3}H]TdR (Amersham, Tokyo) for an additional 24 hr of incubation. Cell cultures were collected with an automated cell harvester and the amount of radioactivity incorporated into the DNA was measured by a liquid scintillation counter. The results of quadruplicate cultures are expressed as c.p.m. $\pm$ SD.

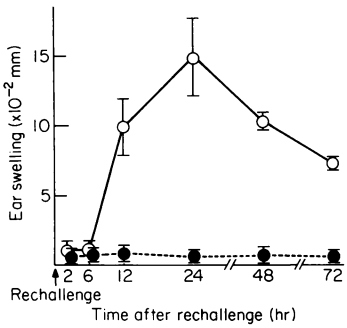

Figure 1. Time–courses of ear swelling on rechallenged site (left ear; ○) and virgin site (right ear; ●) ($n=6$). Mice were sensitized with DNFB on the back on Day 0 and challenged on their left ears on Day 5. Then, on Day 33, they were challenged again with DNFB on their left ears as the rechallenged site and on their right ears as the virgin site. Each ear swelling was measured 2, 6, 12, 24, 48 and 72 hr after the second challenge. Each value represents the mean $\pm$ SEM.

RESULTS

Ear swelling after first challenge and rechallenge, and time–course of ear swelling after rechallenge

Mice were sensitized by the topical application of DNFB on Day 0 and challenged by painting DNFB on the left ear on day 5. We confirmed ear swelling 24 hr after the challenge. Then, on Day 33, challenged again by painting DNFB on both the ears as the rechallenge. Ear swelling after the rechallenge ($23{\cdot}4 \pm 1{\cdot}0$, $n=6$) was considerably stronger than that after the first challenge ($8{\cdot}8 \pm 1{\cdot}0$, $n=6$) ($P<0{\cdot}01$). A peak response of left ear swelling was observed at 24 hr after the rechallenge. No significant response was observed on the right ear (Fig. 1). This result suggests that this left ear swelling was due to DTH reaction. Ear swelling was therefore measured at 24 hr in light of the present experiments.

Rechallenge at the previously CH-responded site and virgin site, and antigen specificity of rechallenge reaction

Next, we adopted the protocol shown in Fig. 2. The DTH response was elicited after the rechallenge with DNFB on the previously CH-responded site to DNFB, whereas no response was induced on the virgin site (Fig. 2, Group 1). When mice were rechallenged with PCl at the previously CH-responded site to DNFB, ear swelling was very low (Fig. 2, Group 2). When mice were rechallenged with A/O only, to clarify whether the previously painted antigen was retained on the ears, no ear swelling was observed (Fig. 2, Group 3). From this observation, it could be suggested that the antigen, in an insoluble form, was not on the ear. It was confirmed that no spontaneous flare-up was observed on the left ear in Group 1 of Fig. 2 during the time interval from the first challenge to the rechallenge. Therefore, it seems that the antigen in immunologically active form did not remain in the ear. When mice were firstly challenged with DNFB on the left ear and with PCl on the right ear on Day 33 after sensitization with DNFB, no response was developed on both ears (Fig. 2, Group 4). These results show that the systemic hypersensitive state has not presented any more by 33 days after the induction of sensitization. No CH response was elicited after

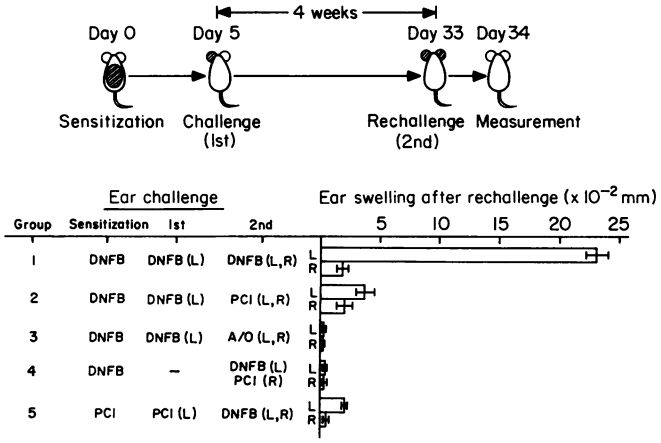

Figure 2. Flare-up of DTH reaction in mice by local application of hapten ($n=6$ in each group). Mice were sensitized with DNFB on the back skin on Day 0, firstly challenged with DNFB on their left (L) ears on Day 5 and secondly rechallenged with DNFB on both ears on Day 33. Then ear swelling was measured 24 hr after rechallenge (Group 1). Mice were sensitized and firstly challenged with DNFB, then, secondly, in one group they were rechallenged with PCl on both their ears (Group 2) and in another group rechallenged with A/O (Group 3). Mice were sensitized with DNFB and firstly challenged with DNFB on the left ear and then with PCl on the right (R) ear 33 days after sensitization (Group 4). Mice were sensitized with PCl, challenged with PCl and rechallenged with DNFB (Group 5). Ear swelling on both ears in each group was measured 24 hr later. The results are represented as mean ± SEM. The left ear swelling in Group 1 is significantly different from others ($P<0.001$).

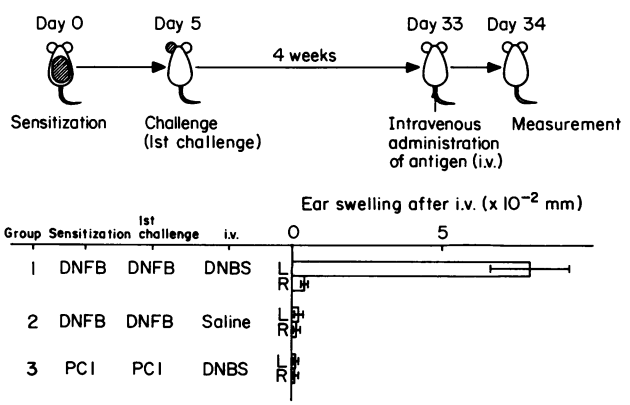

Figure 3. Flare-up of DTH reaction in mice by intravenous administration of hapten ($n=6$ in each group). Mice were sensitized with DNFB and challenged with DNFB on the left ear, then they were intravenously administered DNBS 4 weeks after the first challenge (Group 1). In another group, mice were intravenously injected with saline as a control group (Group 2). Mice were sensitized and firstly challenged with PCl, and intravenously administered DNBS 4 weeks after (Group 3). Ear swelling on both ears in each group was measured 24 hr after the intravenous administration. The results are represented as mean ± SEM. The left ear swelling in Group 1 is significantly different from others ($P<0.001$).

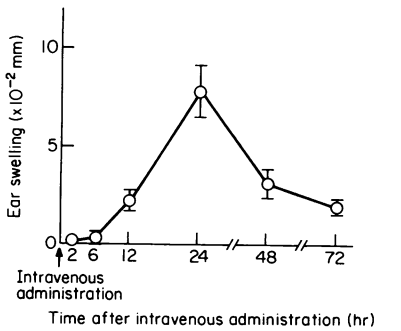

Figure 4. Time-course of ear swelling after intravenous administration of DNBS ($n=6$). Mice were sensitized with DNFB on the back skin on Day 0 and challenged with DNFB on the left ear on Day 5. Then they were intravenously injected with DNBS 4 weeks after the challenge. Ear swelling on their left ears was measured 2, 6, 12, 24, 48 and 72 hr after the intravenous administration. Each value represents the mean ± SEM.

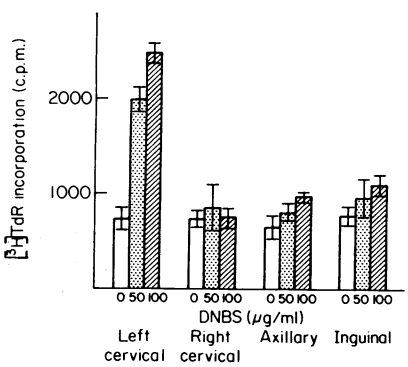

Figure 5. Lymph node cell proliferation to DNBS. Mice were sensitized with DNFB on Day 0 and challenged with DNFB on Day 5. Then on Day 33, left cervical, right cervical, axillary and inguinal lymph nodes were removed and dissociated into single cell suspension. These cell suspensions were incubated with or without DNBS. Culture were carried out for 4 days and pulsed with [^{3}H]TdR for additional 24 hr of incubation. The cells were harvested using an automated harvester and the radioactivity was determined in a liquid scintillation counter. The results of quadruplicate cultures are expressed as the mean c.p.m. ± SD.

the rechallenge with DNFB in the mice in which CH reaction to PCl was elicited once before on the left ear (Fig. 2, Group 5). These results suggest that the ear swelling induced by the rechallenge was an antigen-specific response, and remarkable ear swelling after the rechallenge was elicited only on the first challenged site with the same antigen as used in the sensitization and in the first challenge.

Intravenous administration of antigen

In the next experiment, mice were challenged by the intravenous administration of DNBS as the second challenge on Day 33 (Fig. 3). Ear swelling was elicited on the left ear of prior CH reacted mice to DNFB mice, and no swelling was found on the right ear, the virgin site (Fig. 3, Group 1); a peak response of this ear swelling was observed 24 hr after the intravenous adminis-

tration (Fig. 4). There was no swelling on both the ears after the intravenous administration of saline (Fig. 3, Group 2). There was also no reaction on the previously CH-responded site to PCl after the systemic rechallenge with DNBS (Fig. 3, Group 3). These results indicate that ear swelling after the systemic administration of antigen is also due to DTH reaction and this response shows antigen specificity. Hence, it could be suggested that there is a certain local immunological memory to the specific antigen on the previously CH-responded site to the antigen.

Lymph node cell proliferation

Figure 5 shows that the cells from the left cervical lymph node were enhanced to proliferate by DNBS *in vitro*, which was added into the culture medium. The cells from the right cervical, axillary and inguinal lymph nodes were hardly proliferated by the same antigenic stimulation *in vitro*. This result suggests that there are memory lymphocytes to the specific antigen in the regional lymph node of the previously CH-responded site.

DISCUSSION

In the present study, it was found that a marked ear swelling occurred only on the first challenged site 5 days after sensitization, when the same antigen was applied again 4 weeks later. The ear swelling was found to show its peak at 24 hr after the rechallenge. In the CH reaction of the mouse it has been reported that ear swelling after challenge on the fifth day of sensitization is a DTH reaction, showing its peak 24–48 hr after challenge (Claman *et al.*, 1980a; Asherson & Ptak, 1968), and it is known that the T cell is the principal cell responsible for the DTH reaction (Crowle, 1975; Chase, 1976; Moorhead, 1978; Claman *et al.*, 1980b). Hence, the reaction after the rechallenge in this experiment is also considered to be a DTH reaction due to cellular immunity, mainly related to T cells.

Natsuaki *et al.* (1989) investigated the DNFB CH reaction in BALB/c mice, and found marked ear swelling only when the mice were challenged 5 days after sensitization. They reported that ear swelling was hardly noted if the mice were challenged more than 1 week after sensitization. They also reported that the *in vitro* lymphocyte proliferative reaction to the specific antigen was strongest with the lymph node cells from the mice 5 days after sensitization. In this experiment, since ear swelling was not observed in the right ear in Group 1 of Fig. 2, it seemed that the systemic hypersensitivity was lost by 4 weeks after the first challenge in these mice. Despite the loss of systemic hypersensitivity, marked ear swelling was noted in the same site as the previously swollen ear after the rechallenge (Fig. 2, Group 1), and it was confirmed that this ear swelling showed antigen specificity (Fig. 2, Groups 2–5). Furthermore, due to the fact that the ear swelling by the rechallenge was much stronger than that by the first challenge, it is considered that a location which has once shown a hypersensitive reaction is ready to develop inflammation following exposure to even a slight quantity of the antigen.

In the tuberculin skin reaction, it has been reported that the sites at which positive tuberculin skin tests have been elicited exhibit an accelerated reaction upon reinjection of tuberculin (Arnason & Waksman, 1963; Van Maarsseveen, Bomhof & Scheper, 1982). Also, in the study of CH reaction, Nakagawa *et al.* (1978) investigated the retest reaction in CH to DNCB, and reported that DNCB-sensitized guinea-pigs demonstrated an accelerated reactivity on retest with DNCB at the site of prior CH reaction. Furthermore, Uesugi *et al.* (1985), in an experiment on murine CH reaction to oxazolone, reported that an accelerated antigen-specific reaction after challenge with oxazolone was elicited in the retest site. They also reported that there was no difference in the frequency of epidermal Langerhans' cells between the retest site and the virgin site. It is considered that epidermal Langerhans' cells can not be related to the locally accelerated CH reaction.

Next, we tried to induce such flare-up reaction of DTH by systemic administration of the antigen instead of local administration. Ear swelling was noted again only on the ears which have once developed a hypersensitivity reaction, while no reaction was observed in the other ears (Fig. 3). This ear swelling was also a DTH reaction, reaching its peak in 24 hr (Fig. 4), as was the ear swelling caused by the local application of antigen. From these results, it appears that cells specifically reacting to the antigen remain in the location which has once developed a hypersensitivity reaction, and these cells were estimated to be T lymphocytes.

Klasen *et al.* (1987), on administering antigen locally or systemically after injecting cloned helper T cells into the hind feet of mice, reported that a flare-up of the local DTH reaction was observed. Scheper *et al.* (1983) reported hapten-specific T lymphocytes remained for several months in the locations once affected by CH in guinea-pigs, and these reports seem to support our contention.

Concerning the reactivity of the lymphocytes to specific antigen *in vitro*, Natsuaki *et al.* (1989) have reported that the intensity of ear swelling and the degree of proliferative reaction of lymphocytes correlated with each other. In the present study, even in the mice which have lost systemic hypersensitivity, the lymphocytes from the left cervical lymph node showed a proliferative reaction to the specific antigen. It is therefore suggested that cells reacting to the specific antigen also remained in the regional lymph nodes of the ear on which CH reaction was elicited previously.

From the present results, we consider that memory T cells to the specific antigen remain for a long period in the previously CH-responded skin site and its regional lymph nodes. Therefore, it could be thought that local memory T cells would be activated by specific antigenic stimulation brought about either locally or systemically, and release various lymphokines, resulting into a marked hypersensitivity reaction.

Relating these results to clinical conditions, local recurrent reactions in chronic contact dermatitis or fixed drug eruptions are estimated to be caused by the prolonged presence of antigen-specific memory T cells in the locations which have previously developed allergic inflammation.

Accordingly, this 'rechallenge system' seems to be an important model for elucidating the pathogenesis of intractable contact dermatitis and similar diseases.

REFERENCES

ARNASON B.G. & WAKSMAN B.H. (1963) The retest reaction in delayed sensitivity. *Lab. Invest.* **12**, 737.

ASHERSON G.L. & PTAK W. (1968) Contact and delayed hypersensitivity in the mouse. I. Active sensitization and passive transfer. *Immunology*, **15**, 405.

CHASE M.W. (1976) Developments in delayed-type hypersensitivities: 1950–1975. *J. Invest. Derm.* **67**, 136.

CLAMAN H.N., MILLER S.D., CONLON P.J. & MOORHEAD J.W. (1980a) Control of experimental contact sensitivity. *Adv. Immunol.* **30**, 121.

CLAMAN H.N., MILLER S.D., SY M.S. & MOORHEAD J.W. (1980b) Suppressive mechanisms involving sensitization and tolerance in contact allergy. *Immunol. Rev.* **50**, 105.

CROWLE A.J. (1975) Delayed hypersensitivity in the mouse. *Adv. Immunol.* **20**, 197.

DAHLBACK K. & MOLLER H. (1981) Flare-up of contact dermatitis to picryl chloride in the mouse. *Acta Derma. Venereol. (Stockh)*, **61**, 342.

KLASEN I.S., VAN DER KWAST H., DONSELAAR I.G., LADESTEIN R.M.T. & BENNER R. (1987) Flare-up of delayed-type hypersensitivity initially induced by murine cloned helper T cells. *Cell. Immunol.* **108**, 235.

LENS J.W., VAN DEN BERG W.B., VAN DE PUTTE L.B.A. & VAN DEN BERSSELAAR L. (1984) Flare-up of antigen-induced arthritis in mice after challenge with oral antigen. *Clin. exp. Immunol.* **58**, 364.

MOORHEAD J.W. (1978) Tolerance and contact sensitivity to DNFB in mice. VIII. Identification of distinct T cell subpopulations that mediate *in vivo* and *in vitro* manifestations of delayed hypersensitivity. *J. Immunol.* **120**, 137.

NAKAGAWA S., FUKUSHIRO S., GOTOH M., KOHDA M., NAMBA M. & TANIOKU K. (1978) Studies on the retest reaction in contact sensitivity to DNCB. *Dermatologica*, **157**, 13.

NATSUAKI M., YAMASHITA N., HIGASA M. & SAGAMI S. (1989) DNFB contact hypersensitivity in BALB/c mice. *Hifu*, **31**, 172 (in Japanese with English summary).

SCHEPER R.J., VON BLOMBERG M., BOERRIGTER G.H., BRUYNZEEL D., VAN DINTHER A. & VOS A. (1983) Induction of immunological memory in the skin. Role of local T cell retention. *Clin. exp. Immunol.* **51**, 141.

UESUGI T., SEMMA M. & HIGUCHI H. (1985) Locally accelerated contact sensitivity reaction after repeated challenge in mouse. *Hifu*, **27**, 1148. (in Japanese with English summary)

VAN DE PUTTE L.B.A., LENS J.W., VAN DEN BERG W.B. & KRUIJSEN M.W.M. (1983) Exacerbation of antigen-induced arthritis after challenge with intravenous antigen. *Immunology*, **49**, 161.

VAN MAARSSEVEEN A.C.M.T., BOMHOF G. & SCHEPER R.J. (1982) Demonstration of accelerated and increased migration inhibition factor release *in vivo* in a PPD retest reaction. *Int. Archs Allergy appl. Immunol.* **67**, 1.