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Delayed-type hypersensitivity responses regulate collagen deposition in the lung

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SUMMARY

A previous report showed that hamsters immunized by epicutaneous application of 2,4,6-trinitrochloro-1-benzene (TNCB) were susceptible to the development of pulmonary interstitial fibrosis (PIF) if challenged in the lung with the water-soluble form of this hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS). In this study, we investigated the immunological mechanisms that contributed to increased collagen content in the lungs of hapten-immune hamsters after receiving a pulmonary challenge of the sensitizing hapten trinitrophenol (TNP). In order to evaluate the concept that delayed-type hypersensitivity (DTH) reaction modulated their response to TNP in the lung such that it eventuated into PIF, we compared the cutaneous DTH response (48 hr after challenge) with lung collagen deposition (14 days after challenge) in several lines (strains) of hamsters. The inbred LSH strain, was a high responder in the DTH assay to TNP and developed non-resolving PIF in the hapten-immune animals. This is called hapten-immune pulmonary interstitial fibrosis or HIPIF. We also observed that female LSH hamsters were more susceptible to HIPIF induced by TNP than males. On the other hand, age factors influenced DTH and PIF in random-bred LVG hamsters since young hamsters (3 months old) were low responders to TNP and did not develop PIF in the HIPIF model but matured LVG hamsters (retired breeders) possessed DTH reactivity to TNP and subsequently developed PIF. These results suggest that lung collagen deposition in hapten-immune hamster is regulated by T-lymphocyte-mediated immune inflammation (DTH) in the lung and both are dependent on the ability to develop a cutaneous DTH reaction to the hapten. The elucidation of possible mechanisms of DTH-mediated non-granulomatous, non-resolving PIF is important for understanding of the role of environmental chemicals similar in action to haptens in the mediation of skin and lung diseases.

INTRODUCTION

It was noted previously that chronic inflammation in the lower respiratory tract, especially alveolitis, has a central role in the development of pulmonary interstitial fibrosis (PIF).[1] This chronic alveolitis can give rise to the injury that leads to alveolar dysfunction and eventual loss of the capacity of the lung to exchange gas. However, the mechanism that induces alveolitis is not well understood. A number of studies have used animal models[2-5] to investigate T lymphocytes in the development of PIF, but the precise role of the T lymphocyte in these models is still controversial. For example, Pigeut and colleagues have demonstrated that T lymphocytes have a central role in developing PIF induced by bleomycin,[3] but not induced by silica.[5] In contrast to their report, Kumar *et al.* showed that T lymphocytes participate in PIF induced by silica.[6] As we reported in a previous paper, hapten-immune hamsters that were skin sensitized with 2,4,6-trinitrochloro-1-benzene (TNCB) and then intratracheally challenged with 2,4,6- trinitrobenzene sulphonic acid (TNBS) developed pulmonary inflammation and non-resolving PIF.[7] On the other hand, hamsters challenged with only TNBS developed resolving PIF similar to that induced by bleomycin.[8] The hapten-immune pulmonary interstitial fibrosis (HIPIF) model is different from animal models of delayed-type hypersensitivity (DTH)-induced granulomatous lung disease[9,10] because of the absence of granulomatous lesions when lung tissue is examined histologically. However, the development of fibrosis in the HIPIF model is antigen specific since unrelated hapten (DNP) given intratracheally to TNF-sensitized animals did not produce long-lasting fibrosis.[7] The purpose of the current experiment was to determine if the

Abbreviations: CS, contact hypersensitivity; DTH, delayed-type hypersensitivity; HIPIF, hapten-immune pulmonary interstitial fibrosis; IFN-γ, interferon-γ; IL-2, interleukin-2; i.t., intratracheal; PBS, phosphate-buffered saline; PIF, pulmonary interstitial fibrosis; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TNCB, 2,4,6-trinitrochloro-1-benzene; TNF-β, tumour necrosis factor-β; TNP, trinitrophenol.

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ability of various hamster strains to respond to the immunization and challenge with TNP by developing prolonged lung inflammation and eventual PIF correlated with the ability of that strain to express a cutaneous DTH response to the hapten.

MATERIALS AND METHODS

Animals

Male and female inbred MHA, LSH hamsters and female random-bred LVG hamsters were used. The MHA and LSH hamsters used were young adult age (3–6 months) and LVG hamsters used were young adults or matured retired breeders (older than 6 months). The animals used were maintained according to NIH guidelines under the supervision of the Division of Veterinary Resources and received pelleted food and water *ad libitum*.

Antigens and immunization

The TNCB was purchased from Matheson Coleman Bell Co. (Cincinnati, OH); TNBS was purchased from Eastman Kodak Co. (Rochester, NY).

For skin sensitization, hamsters were anaesthetized with ether. The abdomen was shaved with electric clippers and the remaining stubble was removed with a razor blade. One hundred microlitres of 7% TNCB (7 mg) in carrier (acetone:olive oil = 4:1) was applied to the shaved abdomen and spread locally with the pipette tip.

Intratracheal challenge

Five days post-sensitization with the TNCB, immune and non-immune animals were anaesthetized with 3% chloral hydrate and challenged by intratracheal inoculation of 100 μl of 3% TNBS (3 mg) in phosphate-buffered saline (PBS) (pH 8·0).[7] Visualization of the placement of the cannula in the trachea was accomplished through a surgical incision on the middle of the neck.

Elicitation of contact hypersensitivity

The ears of the hamsters were challenged on the dorsal surface with 20 μl of 1% TNCB in carrier as previously described.[11] Ear swelling was measured at 24, 48 and 72 hr with an engineer's micrometer. The change or increase in swelling was considered positive if the mean swelling was statistically different from the mean swelling of the unimmunized negative control group.

Histological examination

Haematoxylin and eosin stained slides were prepared from lungs that had been previously inflated and fixed 10% buffered formalin. Each section was paired with a paraffin-fixed slide stained with Masson's trichrome for definitive identification of collagen. Representative areas were selected for micrographs. Semi-quantitative analysis of the pathological changes in the MHA strain was reported previously.[7]

Assessment of inflammation

Two weeks post-intratracheal challenge for TNBS, the animals were killed by an overdose of sodium pentobarbital. Lung inflammation was best analysed at 2–5 days post-challenge. Here, the prolonged lung inflammation was assessed by measuring lung index after 2 weeks.[12,13] Increases in lung weight indices, which indicate pulmonary inflammation, were determined by the following equation:

$$\text{lung index} = \frac{\text{lung weight/body weight (experimental)}}{\text{lung weight/body weight (control}}$$

Control syngeneic hamsters were inoculated intratracheally with PBS or not as indicated in Results.

Total hydroxyproline content in the lung

Because the vast majority of hydroxyproline in the lung is found in collagen,[14] the quantification of lung hydroxyproline provides a reasonable index of lung collagen content. The lungs were excised, minced with fine scissors, and hydrolysed in 10 ml of 6 N HCl. Aliquots of the hydrolysate were placed in glass tubes (16 × 150 mm) and the samples were evaporated and resuspended in water. The hydroxyproline in the sample was assessed colorimetrically at 557 nm using *p*-dimethylamino-benzaldehyde.[15]

Statistical analysis of the data

The results are expressed as mean ± SEM. Statistical significance was determined by the Student's unpaired *t*-test, with significance taken as $P < 0.05$.

RESULTS

Induction of contact hypersensitivity (CS) to TNCB

As a first step towards testing our hypothesis that only those hamster strains that responded to TNP with a positive CS (DTH) response would develop PIF when challenged in the lung, we surveyed inbred and outbred hamsters for their DTH reactivity to TNP. TNCB in carrier was applied to the shaved abdomen of MHA, LVG, or LSH hamsters as described in Materials and Methods and previously.[7] Five days later, ears are challenged with the immunizing hapten and ear swelling was measured with a micrometer at 24, 48 and 72 hr. The peak swelling was recorded at 48 hr and is shown here. We observed that LSH, MHA and matured, but not young, LVG hamsters responded to skin sensitization and challenge with a specific increase in swelling (Fig. 1). These observations support the work by Sullivan *et al.*, that reported that LSH and MHA respond to TNP with CS and expand these data by showing that the LVG response is dependent on the age of the hamster.

The hydroxyproline content in the total lung

We were interested in determining if those animals that responded with DTH reaction also showed an increase in collagen deposition in the lung 14 days after intratracheal challenge with TNBS. The MHA and LSH inbred strains share the same major histocompatibility complex (MHC) allele (Hm-Ia) but differ at a few minor histocompatibility loci and they also share the same immune response gene (IR) BSA allele.[16] In general, both strains responded similarly to a variety of immunogens.[17] The lung collagen deposition was assesed by measuring the hydroxyproline content in the total lung.[7] Hapten-immune LSH hamsters produced a significant ($P < 0.05$) increase in total lung collagen when results were compared with hydroxyproline content from challenged-only animals (Table 1, Fig. 2). This result was similar to total increase

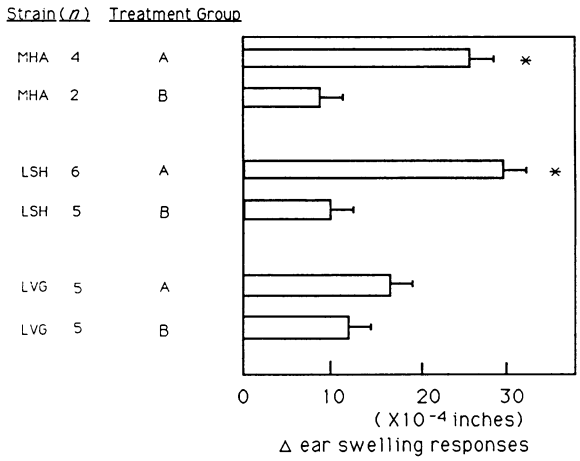

Figure 1. Strain distribution pattern of DTH to TNP. Inbred strains MHA, LSH and LVG random-bred Syrian hamsters were skin painted with 7% TNCB (group A). All were ear challenged 5 days later with 1% TNCB. Bars represent mean maximal ear swelling responses for each group ± SEM. Values for experimental groups (group A) indicated with an asterisk (*) are significantly different from appropriate negative controls (group B) ($P \leqslant 0{\cdot}01$).

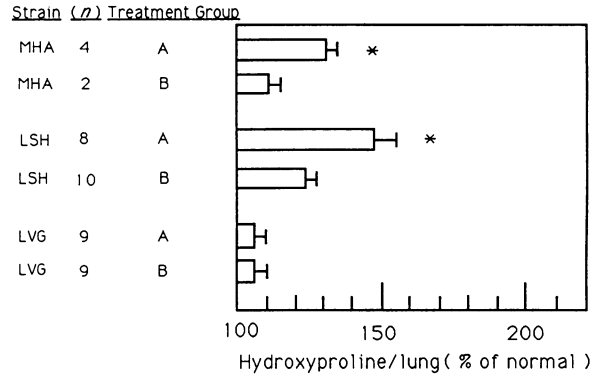

Figure 2. Strain distribution pattern of hydroxyproline in the lung. Inbred strains MHA, LSH and LVG random-bred Syrian hamsters were skin painted with 7% TNCB (group A). All were i.t. challenged 5 days later with 3 mg (100 μl, 3%) of TNBS in their lungs. Intratracheal inoculation only animals are expressed as group B. Fourteen days after i.t. challenge with 3% TNBS, animals were killed and total lung hydroyproline content was measured. Bars represent mean hydroxyproline content for each group ± SEM. Values for experimental groups (group A) indicated with an asterisk (*) are significantly different from appropriate negative controls ($P < 0{\cdot}05$).

of collagen when the MHA strain was used.[7] In addition, histologically, 14 days after i.t. challenge with TNBS, pulmonary lesions in hapten-immune LSH hamster were similar to previous reports for the MHA hamster.[7] In contrast, lungs from hapten-immune young adult LVG hamsters contained little or no increase in hydroxyproline compared to controls. These results support our working hypothesis that the ability to respond to TNP with a DTH response is critical for the development of pulmonary fibrosis in this model. In other words, if the animal is unable to mount a cutaneous DTH response to the immunizing hapten (TNP), it will be saved from HIPIF lesion.

The effect of age on DTH and lung collagen deposition

It is known that a maturation of the DTH response in both mice[18] and rats[19] may vary with various strains. As reported, young LVG hamsters expressed neither a DTH skin response nor HIPIF response to immunization and challenge to TNP. It was therefore important to determine if this inability to respond to TNP changes with age. Interestingly, we observed that LVG hamsters acquired DTH and PIF responsiveness to TNP as they grew older (Fig. 3). A significant amount of collagen was deposited in the lung of hapten-immune matured LVG hamsters. This result further supports the postulate that DTH response is indispensable in the development of non-resolving fibrosis.

Table 1. Lung collagen deposition in hapten-sensitized lung challenged LSH hamsters

Group	Treatment*†	No.	Hydroxyproline ± SEM (μg/lung)	% of control
A	Skin 7% TNCB, i.t. 3% TNBS	12	1175·3 ± 39·5	146·8*
B	Skin none i.t. 3% TNBS	12	1027·0 ± 33·2	128·3
C	Normal	10	800·6 ± 32·5	100·0

† LSH hamsters, matched for age and sex, were skin sensitized with TNCB on Day 0 and i.t. challenged with TNBS on Day 5 as described in Materials and Methods. The hydroxyproline content in the total lung was measured 14 days after i.t. challenge.

* $P \leqslant 0{\cdot}05$ (A versus B).

The effect of ear challenge on HIPIF model

In an effort to study further the relationship between DTH response to TNP and the subsequent lung collagen deposition we evaluated both ear swelling and PIF in the same animal.

LSH hamsters were immunized on the skin and challenged on Day 5 both on the ear and the lung. Challenged-only animals also were challenged on the ear and the lung. All sensitized animals responded positively to the ear challenge and all animals that received the i.t. challenge responded with similar amounts of hydroxypoline regardless of whether the ear had been challenged or not (Table 2).

The absolute amount of hydroxyproline measured in lungs of ear and i.t. challenged was compared to the hydroxyproline content of the lung from challenged-only hamsters. We concluded that the additional challenge did not alter the outcome of the i.t. challenge.

The effect of sex on DTH and fibrosis

To evaluate further the relationship between cutaneous DTH and hydroxyproline, we examined the effect of sex on the animal's ability to respond to TNP either on the skin or in the lung. Essentially, no difference was observed in cutaneous DTH response between male and female LSH hamsters (data not shown). But, interestingly, female hamsters produced more

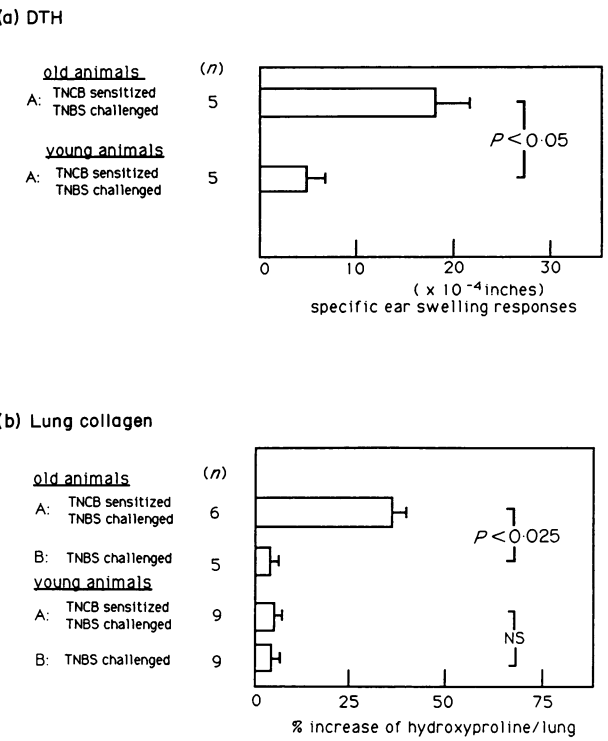

Figure 3. Effect of age on DTH to TNP and lung collagen deposition in LVG hamsters. Young (3 months old) and old (10 months old) LVG hamsters were used. Group A animals were immunized and challenged, group B were challenged only. Specific ear swelling = Δ ear swelling (TNCB sensitized) − Δ ear swelling (control TNCB challenged only); % increase of hydroxyproline = hydroxyproline in experimental animals ÷ hydroxyproline control animals (NS, not significant).

collagen in the lung than male hamsters ($P<0{\cdot}0005$) (Fig. 4). These results suggest that similar to human fibrotic diseases there are multiple factors that may effect the development of PIF in this model.

DISCUSSION

In summary, we have evaluated the mechanisms that are associated with the induction of hapten-immune pulmonary interstitial fibrosis, in hamsters. We showed that the lung collagen deposition induced by the inoculation of hapten (TNBS) into the lung in hapten-sensitized hamster was closely associated with cutaneous DTH responsiveness to the same hapten and correlated with the appearance of immune-mediated inflammation in the lung of the hapten-immunized animal. In addition, the data reported show that other factors such as age and sex may contribute to the increase in collagen deposited if the fibrosis persists. To our knowledge, this report is the first to demonstrate the effects of age and sex on pulmonary fibrosis mediated by DTH (CS) responses to soluble antigens. These data also support the hypothesis that cell-mediated immune mechanisms can promote a pulmonary interstitial fibrosis that resists healing.[7] Whether the immune response actually prevents healing or promotes scarring is yet to be determined.

It is well known that DTH reactions are examples of T-cell-mediated immunity *in vivo*. In DTH, local challenge with antigen elicits an antigen-specific delayed inflammatiaon reaction that depends on the ability of sensitized thymic-derived lymphocytes to recruit non-immune, blood-borne cells in the extravascular tissues .[20] It is alwo well known that TNP has a strong capacity to elicit contact hypersensitivity reaction in several hamster strains.[11] In a previous paper,[21] we showed immunization of TNP by the i.t. route was sufficient to sensitize hamster for contact hypersensitivity response in the skin. A later paper established the animal model of pulmonary interstitial fibrosis induced by this hapten.[7] On the other hand, DTH reactions to TNP in the lungs of mice were reported by Enander *et al.*,[22] and Garssen and colleagues.[23] These investigators

Table 2. Effect of ear challenge with TNCB on hydroxyproline in the lung

Group*	No.	Sensitized	Challenge i.t.	Challenge Ear	Hydroxyproline† (mg/lung) ± SEM
A	3	+	+	−	1·19 ± 0·12
A′	6	+	+	+	1·14 ± 0·13^{NS}
B	2	−	+	−	1·05 ± 0·15
B′	3	−	+	+	1·06 ± 0·16^{NS}
C	2	−	−	−	0·86 ± 0·10
C′	8	−	−	+	0·79 ± 0·09^{NS}

* Female LSH hamsters (6 months old) were used. Animals in group A, A′ were skin sensitized with 10 μl of 7% TNCB on Day 0, group B, B′ were not sensitized but challenged like A, A′ with 100 μl of 3% TNBS. Group C,C′ were not sensitized or i.t. challenged. A′,B′,C′ groups were challenged on the ear on Day 5 with 20 μl of 1% TNCB.

† Fourteen days after i.t. challenge lungs were processed for biochemical studies and the mean hydroxyproline content (± SEM) was calculated.

^{NS} Not significant (versus ear unchallenged groups).

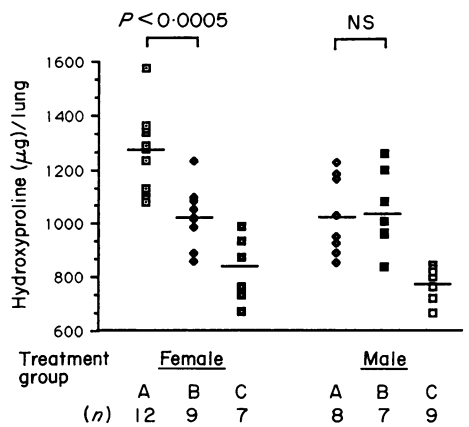

Figure 4. Effect of sex on lung collagen deposition to TNP. The LSH hamsters (6 months old) were used. The animal treatment groups A, B and C, were immunized and challenged as described in Fig. 3. Fourteen days after i.t. challenge with 3% TNBS, total lung hydroxyproline content was measured. Bars represent the mean values in each group (NS, not significant).

concluded that DTH responses in the lung were histologically similar to the cutaneous DTH.[23]

The general concept that fibrosis develops because of prolonged inflammation and associated tissue injury[24] may explain why the i.t. route for antigen challenge to hapten inoculated into the lung induced fibrosis. Interestingly, Oite *et al.* reported that DTH response to TNP by itself produced tissue injury and was capable of modulating successive glomerular disease[25] and raised the possibility that cell-mediated immunity may play a role in interstitial nephritis.[26] It follows that the DTH-mediated mechanisms that induce scarring in the lung in the hapten-immune model may be a representative mechanism that leads to scar tissue formation in a variety of tissues.

Recently, we reported that TNF activity was increased in the lung of all hapten-challenged hamsters but only sensitized hamsters that were challenged released interleukin-2 (IL-2) and tumour necrosis factor-β (TNF-β).[27]Other studies showed *in vitro* that one of the helper T-lymphocyte subsets, Th1 lymphocytes, is closely associated with DTH reaction. It has been reported that Th1 lymphocytes release IL-2, TFN-β and interferon-γ (IFN-γ) not only *in vitro* but also *in vivo*.[28] In addition, Zaloom *et al.* showed that systemic administration of IL-2 enhances the DTH reaction.[29] The possibility is raised that the putative Th1 lymphocytes in the lung of hapten-immune animals release IL-2 and other lymphokines then could directly and indirectly affect the various macrophage or fibroblast subpopulations in the lung. Also, a variety of soluble factors from both lymphocytes and macrophages have been shown to affect collagen production in the lung.

In this report we observed the effect of genetics, age and sex factors on the ability of hamsters to develop PIF as well as DTH. The effect of age on DTH response to TNP in mice is well documented,[18] albeit the mechanism of acquisition of DTH responsiveness to the hapten with maturation is not well understood. The young adult LVG hamster, like a young C57BL/6 mouse, lacked the DTH responsiveness to TNP, but acquired responsiveness as it aged. Loss of suppression could explain this phenomenon and could be tested using cyclophosphamide, a method used by Sullivan *et al.* to show T suppressor down-regulation of the DTH response to TNP in BALB/c mice.[30] Furthermore, even in the MHA strain where a PIF response was noted in young animals, there was an ageing effect on the relative amount of hydroxyproline deposited in the lung in the MHA strain.[31] Therefore, we can conclude that age may be one of the important factors of HIPIF.

It is known that sex factors affect DTH responses to TNP in some inbred mouse strains[32] but not all.[18] Furthermore, oestrogen has been reported to suppress DTH response but augment antibody response in some inbred mouse strains.[33] Although the role of antibody-mediated immunity in the development of HIPIF has not been studied, we canot rule out its participiation. In this sense, the observation that female LSH hamsters were more susceptible to pulmonary fibrosis correlates well with clinical observations that women are more prone to develop fibrotic conditions associated with collagen vascular diseases. The exact role of sex hormones in the development of PIF, however, remains to be explored.

In summary, the HIPIF system was used to investigate the immunopathogenesis of T-lymphocyte-mediated fibrosis induced by soluble antigens. The results may be relevant to an understanding of fibrotic conditions in the lung that are associated with collagen vascular disease, sarcoidosis, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis as well as pulmonary fibrosis induced by environmental toxins that might be touched and/or inhaled.

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