Involvement of cytokines in the pathogenesis of hypersensitivity pneumonitis: Evaluation of immunotherapeutic measures in a mouse model

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M DENIS, M BÉDARD, J GAGNON, Y CORMIER, M LAVIOLETTE. Involvement of cytokines in the pathogenesis of hypersensitivity pneumonitis: Evaluation of immunotherapeutic measures in a mouse model. Can J Infect Dis 1992;3(Suppl B):111B-114B. C57BL/6 inbred strain mice were instilled intranasally with 150 µg/day of the actinomycete *Faeni rectivirgula* three days a week as a model of farmer's lung disease. Instilled mice developed a strong alveolitis manifested by a large increase in the number of cells in the bronchoalveolar lavage (BAL) (4.1×10^4 cells in saline controls versus 5.28×10^5 cells in *F rectivirgula*-instilled mice). This influx was associated with a substantial release of pro-inflammatory cytokine in the BAL; 170 U/mL of interleukin (IL)-1 in instilled mice whereas saline instilled mice had undetectable levels of cytokines in the BAL. In addition, pulmonary fibrosis was evident in challenged mice at three weeks (twofold increase in lung hydroxyproline levels). Infusion of a rabbit antimouse tumour necrosis factor-alpha was associated with an almost complete abrogation of all markers of the disease. Also, administration of cyclosporine A (50 mg/kg/day) partially prevented the alveolitis ($P < 0.01$) and totally prevented cytokine release and lung fibrosis in challenged mice. These findings underscore the immunological basis of hypersensitivity pneumonitis, and suggest that antagonism of inflammatory cytokines may hold promise in the treatment of this pathology.

Key Words: Cytokines, Fibrosis, Hypersensitivity pneumonitis

Rôle des cytokines dans la pathogenèse de la pneumopathie immunologique: évaluation de mesures immunothérapeutiques dans un modèle de souris

RÉSUMÉ: Des souris de lignée C57BL/6 pure ont reçu une instillation intranasale de 150 µg par jour de l'actinomycète *Faeni rectivirgula* trois jours par semaine, pour établir un modèle de poumon du Fermier. Les souris à qui l'instillation fut administrée ont développé une grave alvéolite, caractérisée par une forte augmentation du nombre de cellules présentes dans le liquide du lavage broncho-alvéolaire (LBA) (4.1×10^4 cellules dans les témoins physiologiques contre 5.28×10^5 dans les spécimens recueillis chez les souris instillées au *F rectivirgula*). Cet influx était associé à une libération substantielle de cytokines pro-inflammatoires dans le LBA: 170 U/mL d'interleukine-1 chez les souris instillées au *F rectivirgula*, alors que les souris instillées au salin physiologique présentaient des taux non décelables de cytokines au niveau du LBA. En outre, une fibrose pulmonaire a été rapportée chez les souris instillées au *F rectivirgula* dès la troisième semaine (augmentation du double des taux d'hydroxyproline pulmonaire). La perfusion du facteur de nécrose tumorale a été associée à une abrogation presque complète de tous les marqueurs de la maladie. Également, l'administration de cyclosporine A (50 mg/kg/jour) a partiellement bloqué l'alvéolite ($P < 0.01$) et totalement empêché la libération de cytokines et la fibrose pulmonaire chez les souris soumises à l'allergène. Ces résultats soulignent la base immunologique de la pneumopathie immunologique et suggèrent que l'antagonisme des cytokines inflammatoires représente un traitement potentiel de cette pathologie.

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HYPERSENSITIVITY PNEUMONITIS DESIGNATES A GROUP OF lung inflammatory diseases which represent the response to inhaled actinomycetes (farmer's lung) or to animal proteins (pigeon breeder's lung) (1). The disease is characterized by an intense alveolitis, composed mostly of mononuclear cells, ie, macrophages and CD8+ lymphocytes (2). Chronic exposure to the antigens may result in the formation of loosely formed granulomas and eventual fibrosis (3). Mouse models of this important pathology have been developed based on the intranasal application of actinomycete, and this animal model may yield results of crucial importance for understanding the disease (4). This model is highly representative of human disease as it is characterized by an early neutrophilic infiltrate and a later phase of alveolitis composed principally of macrophages and lymphocytes. Chronic exposure often results in collagen deposition in the lungs, with irreversible consequences.

To deepen understanding of the immune basis of hypersensitivity pneumonitis a mouse model based on intranasal instillation of *Faeni rectivirgula* was studied. The effect of two anticytokine treatments, namely anti-tumour necrosis factor- α (TNF α) antibodies and cyclosporine A (Sandimmune; Sandoz), were evaluated.

MATERIALS AND METHODS

Mice: Pathogen-free C57BL/6 mice weighing 18 to 20 g were purchased from Charles River (St-Constant, Québec).

Faeni rectivirgula: A strain of *F rectivirgula* (also known as *Micropolyspora faeni* or *Saccharopolyspora rectivirgula*) was grown in trypticase soy broth (Difco, Michigan) for six days at 52°C on a shaking incubator. Cells were harvested by centrifugation, homogenized and lyophilized.

Antimouse tumour necrosis factor- α : An immunoglobulin fraction prepared by ammonium sulphate precipitation from the serum of rabbits hyperimmunized with purified murine TNF α was used as the anti-TNF α antiserum. One milligram of antiserum neutralized 6×10^6 U of cytolytic activity of mouse TNF α in an L929 killing assay (5). This antiserum contained less than 0.1 ng of endotoxin per 10 mg of material, as seen in a Limulus amoebocyte assay (Sigma, Missouri). This antiserum did not block the bioactivity of interleukin (IL)-1, IL-2, TNF β or interferon- γ (IFN γ) in specific bioassays. IL-1 activity was measured by the thymocyte coproliferation assay of Mizel and associates (6); IL-2 activity was measured via the induction of proliferation of CTLL-D cells (6); TNF β activity was measured via the lytic activity towards L929 fibrosarcoma cells (6); and IFN was measured by its ability to protect mouse fibroblasts from vesicular stomatitis virus infection (6).

Induction of hypersensitivity pneumonitis: Animals were lightly anesthetized by diethyl ether and given 150

mg *F rectivirgula* antigen in 50 mL of saline transnasally for three successive days a week. Mice were divided into four groups: group 1 received saline only; group 2 received 150 μ g of *F rectivirgula* three successive days a week for three weeks, group 3 received 150 μ g of *F rectivirgula* three successive days a week for three weeks and 2 mg anti-TNF α intraperitoneally weekly, and group 4 received 150 mg of *F rectivirgula* three successive days a week for three weeks and cyclosporine A 50 mg/kg intraperitoneally every day.

Bronchoalveolar lavage cell number: Animals were killed and cells in the bronchoalveolar lavage (BAL) were isolated by repeated lavage of the airways with 5 mL of 0.6 mM EDTA in phosphate buffered saline. BAL fluid was stored at -20°C and cell number assessed by trypan blue counting.

Fibrosis: The total lung hydroxyproline concentration was determined using previously described methods (7). Briefly, lungs were submitted to an acid hydrolysis, the hydrolysate was neutralized, extracted with phenol-chloroform-isoamyl alcohol and hydroxyproline determined by colorimetry.

Interleukin-1 analysis: IL-1 levels were measured by a thymocyte coproliferation assay described in detail by Mizel (8). Briefly, thymocytes from C3H/HeJ mice were obtained and suspended at 10^7 cells/mL in complete RPMI 1640 with 10% fetal calf serum and antibiotics. One hundred microlitres of cell suspension were added to each well of a 96-well microtitre plate. Test samples of IL-1 and antiserum were added to appropriate wells and plates incubated 48 h at 37°C with 5% carbon dioxide and a final 6 h pulse with 1 μ Ci per well tritiated thymidine. Cell-associated radioactivity was measured by beta counting.

Statistical analysis: Statistical analysis of the differences between means was performed using ANOVA, combined with the Newman-Keul test for significance at $P=0.05$.

RESULTS

Alveolitis blocked by anti-TNF α or cyclosporine A:

The histopathological profile of the transnasal instillation with *F rectivirgula* has been described in detail (9). Instillation with *F rectivirgula* is associated with an acute and strong inflammation with an accumulation of an inflammatory infiltrate comprising macrophages, lymphocytes and neutrophils. Histological examination was suggestive of an interstitial pathology affecting mostly the small and medium airways.

Instillation of *F rectivirgula* by the intranasal route led to a strong influx of mononuclear cells in the BAL (Table 1). At three weeks, Giemsa staining revealed that approximately 50% of the cells were macrophages and 50% lymphocytes. As shown in Table 1, infusion of anti-TNF α led to a total abrogation of alveolitis. Administration of cyclosporine A (50 mg/kg/day) was also associated with a significant abrogation of this alveolitis.

IL-1 release blocked by anti-TNF α or cyclosporine A:

As the data in Table 2 suggest, hypersensitivity pneumonitis induced the release of copious amounts of IL-1 in the BAL (170 U/mL), which was nullified by anti-TNF α or cyclosporine A treatments.

Lung fibrosis blocked by anti-TNF α or cyclosporine A:

Collagen deposition in the lungs of different groups of mice were determined. Hypersensitivity pneumonitis provoked a substantial fibrosis in the lungs. Administration of anti-TNF α or cyclosporine A blocked this fibrotic reaction, with levels of hydroxyproline similar to those of saline-instilled mice. Although lung hydroxyproline is not always strictly correlated with an ongoing fibrosis, an increased level of hydroxyproline is certainly always indicative of a lung remodeling process. Control mice were also given control rabbit globulin and a challenge of actinomycete. Results showed that infusion with rabbit globulin had no effect on the inflammation induced by *F rectivirgula*.

DISCUSSION

This report describes immunopathological features of hypersensitivity pneumonitis and their modulation by cyclosporine A and anti-TNF α . Intranasal instillation of *F rectivirgula* provoked a large alveolitis, IL-1 release and lung fibrosis. Previous results with mouse models of hypersensitivity pneumonitis have emphasized the strong alveolitis seen after actinomycete challenge (4). This is consistent with findings in humans where repeated challenge brings about a drastic recruitment of mononuclear cells (10). Present results are also in agreement with results on the ability of cyclosporine A partially to block cellular recruitment (4). Of considerable interest is finding that application of an anti-TNF α antiserum totally abrogates cellular influx. The presence of IL-1 points to an intense inflammation in the lungs of challenged mice. This is an important point inasmuch as an alveolitis is not always representative of a true inflammatory state.

TNF α is a pleiotropic cytokine involved in the genesis and maintenance of inflammatory reactions (11). TNF α activates leukocytes and induces the synthesis of adhesion molecules involved in the recruitment of leukocytes (12). Previous data have shown the crucial involvement of TNF α in the development of lung injuries; after exposure to silica (13), bleomycin (14) and immune complexes (15). Present results with cyclosporine A also emphasize the immunological basis of hypersensitivity pneumonitis inasmuch as the immunosuppressive agent cyclosporine A is thought to act by preventing lymphokine (notably TNF α) expression and release (16). Overall, this suggests an important role for cytokines in the inflammation of hypersensitivity pneumonitis and similar conditions, and a crucial role for TNF α .

It is unclear how TNF α produces the various aspects of hypersensitivity pneumonitis, including alveolitis, IL-1 release and fibrosis. The alveolitis is surely linked

TABLE 1

Bronchoalveolar lavage (BAL) cell number in hypersensitivity pneumonitis and modulation by anticytokine reagents

Group	Intranasal instillation	Treatment	BAL cell number
1	Saline	Saline	4.1x10 ⁴
2	<i>F rectivirgula</i>	None	52.8x10 ⁴
3	<i>F rectivirgula</i>	Anti-TNF α	5.3x10 ⁴
4	<i>F rectivirgula</i>	Cyclosporine A	22.1x10 ⁴ †

Groups 2, 3 and 4 mice were given 150 μ g of *F rectivirgula* intranasally for three days a week for three weeks. Group 3 mice were given 2 mg of anti-TNF α antiserum weekly. Group 4 mice were given 50 mg/kg of cyclosporine A daily. *P<0.01 versus the other three experimental groups; †P<0.01 versus group 2. For all groups, standard deviations were less than 15% (n=6)

TABLE 2

Interleukin (IL)-1 secretion in the bronchoalveolar lavage of hypersensitivity pneumonitis and modulation by anticytokine reagents

Group	Intranasal instillation	Treatment	IL-1 (U/mL)
1	Saline	Saline	Not detectable
2	<i>F rectivirgula</i>	None	170**
3	<i>F rectivirgula</i>	Anti-TNF α	16
4	<i>F rectivirgula</i>	Cyclosporine A	6

Groups 2, 3 and 4 mice were given 150 μ g of *F rectivirgula* intranasally for three days a week for three weeks. Group 3 mice were given 2 mg of anti-TNF α antiserum weekly. Group 4 mice were given 50 mg/kg of cyclosporine A daily. **P<0.001. For all groups, standard deviations were less than 20% (n=7)

TABLE 3

Lung fibrosis in hypersensitivity pneumonitis and modulation by anticytokine reagents

Group	Intranasal instillation	Treatment	Hydroxyproline μ g/lung
1	Saline	Saline	221 $\pm$ 26
2	<i>F rectivirgula</i>	None	418 $\pm$ 46**
3	<i>F rectivirgula</i>	Anti-TNF α	236 $\pm$ 41
4	<i>F rectivirgula</i>	Cyclosporine A	208 $\pm$ 32

Groups 2, 3 and 4 mice were given 150 μ g of *F rectivirgula* intranasally for three days a week for three weeks. Group 3 mice were given 2 mg of anti-TNF α antiserum weekly. Group 4 mice were given 50 mg/kg of cyclosporine A daily. **P<0.001 (n=8)

to the ability of TNF α to induce the expression and release of chemotactic cytokines such as IL-8 (17). TNF α may be involved directly in stimulating fibrosis as TNF α directly stimulates collagenase activity in fibroblasts (18). Alternatively, TNF α may contribute to the fibrosis by stimulating the release of profibrotic molecules such as platelet-derived growth factor or transforming growth factor beta (19).

The exact role played by T cells in the pathology of hypersensitivity pneumonitis in the mouse model is still unclear. It may be that T cells play a role similar to

that ascribed to these cells in bleomycin-induced lung injury (14), where they play a detrimental role by stimulating enhanced TNF α release from alveolar macrophages. The role played by the different cellular mediators in the development of hypersensitivity pneumonitis is still being pursued in the authors' laboratory.

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