Full Length Research Paper

A study of whether immune complexes from symptomatic individuals activate macrophages than immune complexes from asymptomatic individuals

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Pigeon fanciers©lung is an immunologically-mediated lung disease as a result of inhaling pigeon derived materials. High antibody titres are observed for symptomatic and asymptomatic pigeon fanciers. The aim of the present study was to investigate whether immune complexes from individuals with pigeon fanciers' lung were better at activating macrophages than immune complexes from asymptomatic pigeon fanciers. Serum samples were obtained from fifteen individuals with pigeon fanciers' lung, fifteen asymptomatic pigeon fanciers and fifteen non-farming controls. Solid immune complexes were generated with these serum samples with the pigeon derived antigen mucin. The immune complexes were then incubated with macrophage U937 cells. The supernatants of the cell culture were thereafter analysed for tumour necrosis factor-α (TNF-α), interleukin 6 (IL 6), interleukin 1 (IL 1), nitric oxide and catalase as indices for the activation of macrophages. The activation by lipopolysaccharide was used as a positive control. The results showed that the immune complexes from symptomatic pigeon fanciers activated macrophages significantly better than immune complexes from asymptomatic pigeon fanciers (P < 0.05). Immune complexes from non-farming controls exhibited the least propensity in activating the macrophages. The findings suggest that immune complex activation of macrophages could be used to assign farmers to been symptomatic and asymptomatic.

Key words: Pigeon fanciers' lung, macrophages, activation, immune complexes, mucin.

INTRODUCTION

Pigeon fanciers' lung is an immunologically-mediated lung disease as a result of the inhalation of pigeon derived materials (Calvert et al., 1999). The disease was however, reported in scientific literature in 1965 as a recurrent interstitial pneumonitis due to man's exposure to pigeon derived materials (Reed et al., 1965; Barboriak et al., 1965). The inhaled antigens (the pigeon derived materials) provoke a hypersensitivity reaction in the alveoli and bronchioles of susceptible individuals (Bourke and Boyd, 1997). Immune complexes are formed in the alveoli between the inhaled antigens and pre-existing IgGs and this signals the attraction of neutrophils to the site of the inflammation. This condition known as early neutrophil alveolitis may proceed to the proliferation of lymphocytes (mostly the cytotoxic / suppressor subtype, CD8+), which accumulate within the lung parenchyma and alveolar space of sensitised individuals (Calvert et al., 1999; Dakhama et al., 1996). These lymphocytes may mediate tissue damage after prolonged accumulation which can often be irreversible. This condition that is known as pulmonary fibrosis is a common response to various injuries in the lung (Kuwano et al., 2004).

The acute form of pigeon fanciers' lung is the more common form especially in the UK and individuals with this form of the disease experience intense flu-like symptoms of fever, chills, muscle aches, cough and breathlessness, which occurs about 4 - 8 h after antigen exposure (Bourke and Boyd, 1997). It may progress to the chronic form that is characterised by pulmonary fibrosis and lung function might be seriously compromised. Symptoms may include severe weight loss, cough and development of severe breathlessness (Calvert et al., 1999).

The disease does not progress from the acute form to the chronic form readily. Some people may have the initial acute symptoms but may not develop to pulmonary fibrosis even though they are continually exposed to the antigens. Some may develop pulmonary fibrosis without any detectable acute symptoms (Pérez-Padilla et al., 1993). While others who have the acute form, the symptoms may disappear without any treatment. Some people might be in a state of equilibrium with the antigens for many years before the onset of symptoms (Bourke et al., 1989).

However, in some cases, lung function could be seriously compromised with continual antigen exposure while in others, the disease may worsen even though fanciers restrict further antigen exposure (Bourke and Boyd, 1997; Du et al., 1988). The antigens that cause pigeon fanciers' lung especially pigeon bloom particles have been measured and found to be approximately 1 micron in diameter (Boyd et al., 1982) hence they have the propensity of penetrating the distal gas exchanging tubules. Mucin, a highly glycosylated protein, is one of the major antigens found in pigeon intestinal droppings and is known to be one of the principal antigen causing pigeon fanciers' lung by initiating a strong immune response (Baldwin et al., 1998; Hounsell et al., 1996)

Immune complexes normally activate complement through the classical pathway and also activate macrophages though *Fc* binding. The activation leads to the release of chemotactic factors. The net result will be the attraction of neutrophils to the alveoli. In pigeon fanciers' lung, the neutrophils are incapable of phagocytosing the immune complexes which are trapped in the alveolar epithelium. Rather they release reactive oxygen species, lysosomal enzymes and proinflammatory cytokines which are chemotractic. (Denis et al., 1991; Trembly et al., 1993). Even though it is known that pigeon fanciers' lung is an immunologically mediated lung disease caused by the inhalation of antigens of environmental origin, the fact that only a few people become symptomatic out of all that are exposed to the antigens known to cause the disease is indeed puzzling since many develop high antibody titres to the inhaled antigens (Calvert et al., 1999). It is obvious that both symptomatic and asymptomatic individuals develop immune complexes hence both the classical complement pathway and macrophages would be expected to be activated in all cases, but only a limited number of people have symptoms of diseases (Bourke and Boyd, 1997). This may suggest that immune complexes may not be critical in the pathogenesis of the disease. This work investigates whether immune complexes from sympto- matic individuals activate macrophages than immune complexes from asymptomatic individuals.

MATERIALS AND METHODS

Sera

Venous blood was obtained from fifteen individuals with pigeon fanciers' lung, fifteen asymptomatic antibody- positive fanciers and fifteen normal non-farming controls. Venous blood was obtained from all patients (men, > 45 years) as part of their clinical evaluation and written consent. Blood samples were collected into preservation free canisters and sera collected after centrifugation and stored as aliquots at -80°C.

Materials

The cell line U937 was purchased from European collection of cell cultures (Salisbury, UK), fetal calf serum, L-glutamine, phorbol 12- myristate 13-acetate, lipopolysaccharide (from *Escherichia coli* strain 055:B5), extravidin-HRP conjugate were purchased from Sigma Chemicals (USA). Culture media RPMI-1640 was obtained from *in vitro* technologies (UK). Purified rat anti-human TNF-α, rat anti -human IL-6, mouse anti- human IL-1 and the biotylated forms of the cytokines were obtained from pharmingen (Becton Dickinson). Penicillin was obtained from Euroclone (Italy) . All other reagents and chemicals were of analytical grade thus were used without further purification. All buffers were prepared in double glass-distilled water and dilutions were made in RPMI unless otherwise stated.

Propagation of the cell line U937

All procedures were carried out in a cell culture facility unless otherwise stated. One vial of the cell line U937 were grown in RPMI-1640 medium supplemented with 50 mL heat inactivated fetal calf serum, 5 mL of 0.02 M L-glutamine, 5 mL of 100 /mL penicillin, and 5 mL of 100 μg/mL streptomycin (thus known as the complete RPMI medium). Briefly, the vial of the cell line U937 from the liquid nitrogen was allowed to thaw and added to 5 mL of complete RPMI. Five millilitres of RPMI medium was added to the stock and centrifuged at 2500 rpm for 10 mins. They were washed three times in RPMI and re-suspended in 10 mL of complete RPMI and maintained between 2×10^5 and 1×10^6 cells mL^{-1}. The cells were then kept in a humidified incubator maintained at 37°C and gassing up to 5% CO_2.

Generation of macrophages

The cells were re-suspended at 5×10^5 cells mL^{-1} in complete RPMI medium. Two hundred microlitres of cells in medium was added into each well of a 96 well culture plate. Twenty microlitres of phorbol 12-Myristate 13-Acetate (PMA) (100 ng/mL) was added to the cells and kept in the humidified incubator for 48 h.

Preparation of solid phase immune complexes

Solid phase immune complexes were generated between the serum samples and mucin. Nuncimmuno Maxisorp 96 well plates were coated with 100 L of mucin (1: 1000) and incubated overnight at 4°C. Supernatants were removed and 100 L of complete RPMI medium was added to each well. Two hundred microlitres of each sera (1:10) was added to the top wells and double diluted down the plates and incubated for 2 h under room temperature.

Incubation of solid phase immune complexes with macrophages

The supernatants were then removed from the plates (bearing the immune complexes).

The supernatants were also removed from the culture plates bearing U937 treated with PMA. Two hundred microlitres of complete RPMI was added to the wells of the culture plates. The contents of each well were vigorously agitated and 200 L of each content was added to the wells bearing the solid phase immune complexes and incubated overnight at room temperature.

In vitro assay for macrophage activation

The activation of the macrophages was assayed by measuring the production of tumour necrosis factor alpha (TNF-), interleukin 6
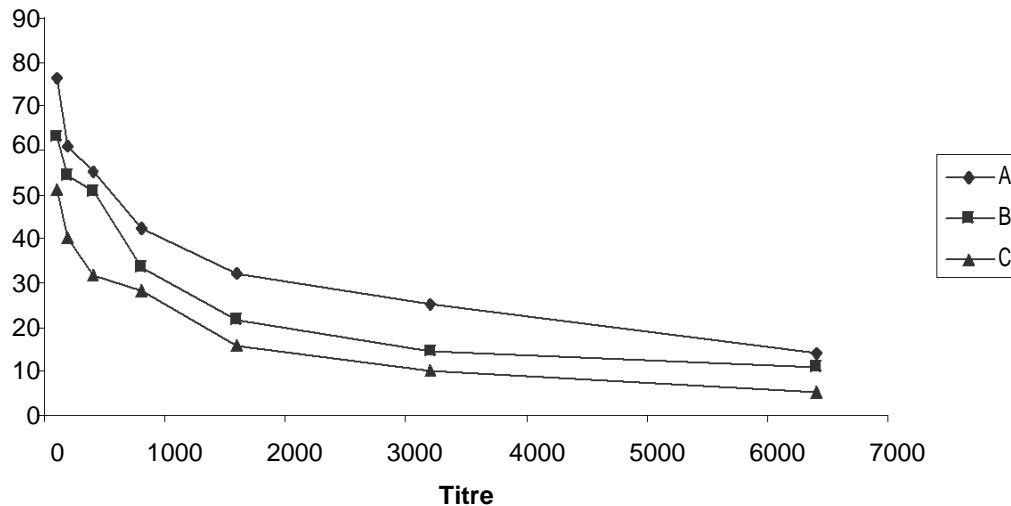
Figure 1. Release of tumour necrosis factor alpha in macrophages activated with solid phase immune complexes generated with mucin and sera from symptomatic pigeon fanciers (A, n = 15), asymptomatic pigeon fanciers (B, n = 15) and non-farming controls (C, n = 15).

(IL-6), interleukin 1 (IL-1), nitric oxide and catalase in the supernatants of the final culture. The cytokines TNF- , IL-6, and IL-1 were determined using cytokine ELISA as reported (Okoko and Oruambo, 2009). Nitric oxide was determined according to the method of Hwang et al. (2002) as modified by Hsieh et al. (2007) Catalase activity was also measured as reported (Okoko and Oruambo, 2009). Lipopolysaccharide (5 ng/mL) was also incubated with some transformed U937 cells as positive control.

Statistical analysis

Lipopolysaccharide-mediated release of the indices from the macrophage U937 cells were arbitrarily assigned 100% and the release mediated by the immune complexes were expressed as percentages of the Lipopolysaccharide-induced releases. For each parameter measured, percentage activation was plotted against titre. Where appropriate, data was subjected to either a Mann-Whitney or a Kruskal -Wallis test. A confidence level exhibited at P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The immune complex mediated release of the cytokines, nitric oxide and catalase were plotted against the titres. As expected, there was a titre dependent release of the indices of macrophage activation. In all cases, the production of the cytokines, nitric oxide and catalase was highest for immune complexes raised against sera from individuals with pigeon fanciers' lung, while the immune complexes of sera from the non- farming controls possessed the lowest activation of the macrophages. In all cases, there were significant differences between the groups (Figures 1 - 5, P < 0.05).

Generally, the exact mechanism involved in the pathogenesis of the disease is not yet known however, it

has been generally accepted that the initial stages that is, the acute stages is immune complex mediated. These immune complexes are formed in the outer alveolar wall as a result of the local reactions between the inhaled antigens and the pre-existing IgGs (Calvanico et al., 1980; Yoshizawa et al., 1988). Hence, these immune complexes can activate complement through the classical pathway to produce anaphylotoxins which will mediate mast cell degranulation, chemotactically attract neutrophils and also stimulate the release of lytic enzymes and reacting oxygen species (Goldsby et al, 2003) hence, there would be persistent inflammation and hypersensitivity. The immune complexes would normally mediate the removal of the antigens by an array of mechanisms including the attraction of phargocytes to digest the immune complexes (Roitt et al., 2002). In the case of pigeon fanciers' lung, these immune complexes may remain in the lung for too long and become deposited in the alveoli and they are not degraded by cells of the reticulo-endothelial system especially neutrophils. In fact, the occurrence of clinically significant type III hypersensitivity reactions is as a result of the formation of large amounts of immune complexes (Tizard, 1994).

However, it is quite puzzling that only a few persons get the disease even though many are exposed to the same antigens that are known to initiate the disease. In this study, the responses of macrophages to immune complexes from symptomatic and asymptomatic pigeon fanciers and also non-farming controls were investigated in order to unravel this anomaly and possibly develop a workable immunodiagnosis.

In this present study, phorbol myristate acetate (PMA) was used to enhance the transition of the monocytic form
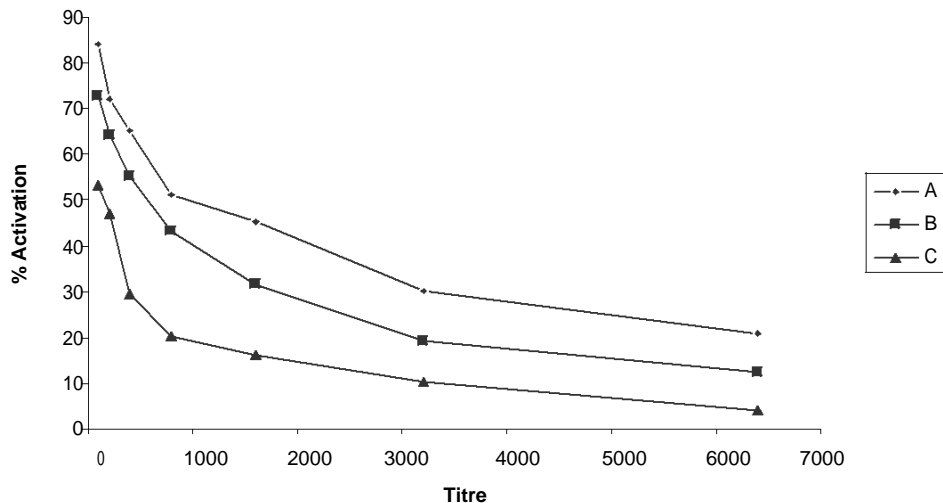
Figure 2. Release of Interleukin 6 in macrophages activated with solid phase immune complexes generated with mucin and sera from symptomatic pigeon fanciers (A, n = 15), asymptomatic pigeon fanciers (B, n = 15) and non-farming controls (C, n = 15).
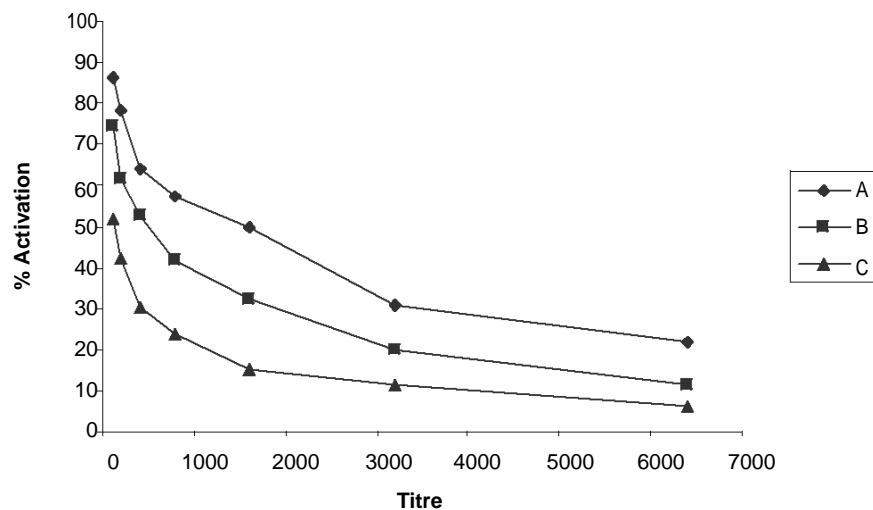

Figure 3. Release of Interleukin 1 in macrophages activated with solid phase immune complexes generated with mucin and sera from symptomatic pigeon fanciers (group A, n = 15), asymptomatic pigeon fanciers (B, n = 15) and non-farming controls (C, n = 15).

of U937 to the macrophage form since it has been shown that phorbol esters in general induce the differentiation of U937 from a round shape to a macrophage-like morphology, from suspension growth and acquire phagocytic activity at concentrations of 8 - 16 ng/mL (Pagliara et al., 2004; Liu and Wu 1992) and the differentiation of the monocyte form of U937 peaks at 48 h (Joyce and Steer, 1992) . The fact that the solid phase immune complexes from the symptomatic individuals seem to activate macrophages more than the immune complexes from the asymptomatic farmers gave further

support to the finding that sera from the symptomatic farmers activate complement better since it is obvious that both processes occur concurrently *in vivo.*

Based on these present observations, immune complex activation of macrophages may be used (if properly developed) to assign patients to been antibody-positive symptomatic farmers and their asymptomatic counter-parts hence, is expected to extend the frontiers of developing an immunodiagnosis for pigeon fanciers' lung. However, neutrophils should also be used in place of macrophages because of their role in phargocytosis.
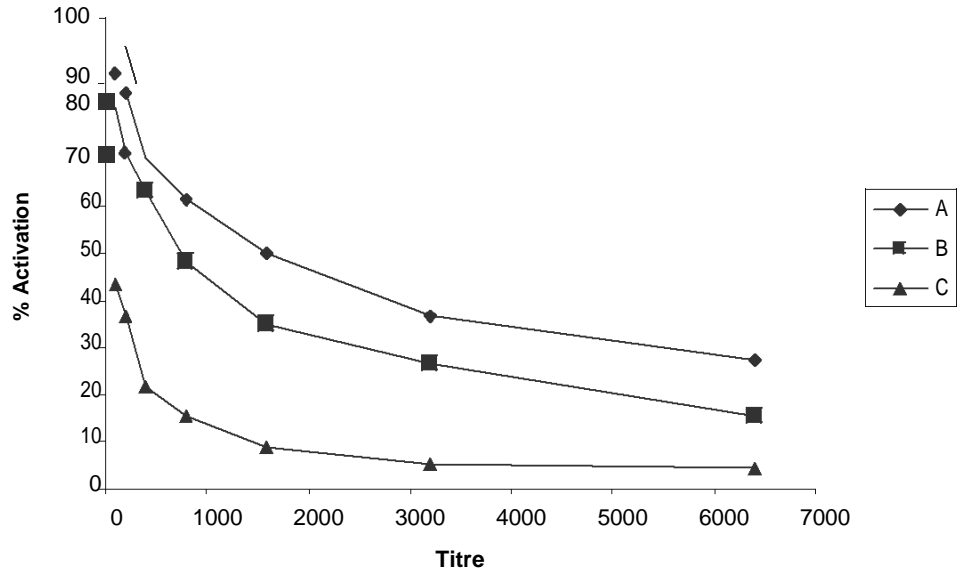
Figure 4. Release of nitric oxide in macrophages activated with solid phase immune complexes generated with mucin and sera from symptomatic pigeon fanciers (A, n = 15), asymptomatic pigeon fanciers (B, n = 15) and non-farming controls (C, n = 15).
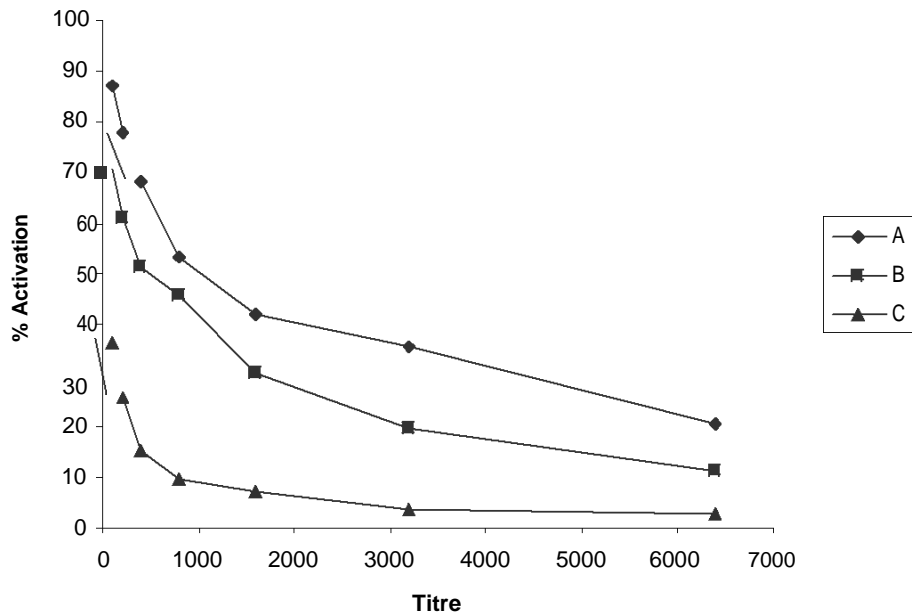

Figure 5. Release of catalase in macrophages activated with solid phase immune complexes generated with mucin and sera from symptomatic pigeon fanciers (A, n = 15), asymptomatic pigeon fanciers (B, n = 15) and non-farming controls (C, n = 15).

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