MACROPHAGE ACTIVATION BY IMMUNE COMPLEX IN PIGEON FANCIER'S LUNG

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ABSTRACT

Pigeon fancier's lung (PFL) is a form of extrinsic allergic alveolitis (hypersensitivity pneumonitis) the pathogenesis of the disease is throught to be caused by the deposition of immune complexes in the alveoli. Although this is generally agreed in the bionic pathogenesis of disease it is difficult to understand why a large proportion of people with high titres of antibody do not get disease. Allergen avoidance to pigeon is the best therapy for Pigeon fancier's lung (PFL). However, allergen avoidance is not possible because of occupational exposure, immunotherapy may be the treatment of choice in cases of pigeon allergy. In this study we firstly showed the optimal concentration of LPS and the optimum time for activation was 10μ/ml for 24 hours after stimulated the cells by PMA for 72 hours. Immune complexes were generated with mucin, fresh pigeon droppings (PDF), old pigeon droppings (PDO), and patents sera. Immune complexes with PDF and PDO activated macrophages to produce TNFα. However immune complex with mucin did not activate macrophages. There was difference in the ability of immune complexes from symptomatic and asymptomatic individual to activate macrophage.

KEYWORDS: Pigeon fancier's lung, Type-III hypersensitivity reactions, Immune complex disease, Allergic disease, Macrophage activation, Pro-inflammatory cytokines.

INTRODUCTION

Pigeon Fancier's Lung (PFL) is a form of extrinsic alveolitis (hypersensitivity pneumonitis)[1,2,3]. The disease was described in the mid-1960, as a recurrent interstitial pneumonitis detection in people who breathed in pigeon-derived material and was thought to be of allergic origin and similar to disease such as farmers lung[1]. PFL is caused by type-III hypersensitivity reactions and thus an immune complex disease[1]. PFL is a rather dynamic heterogeneous clinical syndrome and has two forms. The acute form of the disease which is characterized by recurrent fever. Chills, myalgia and cough whilst the chronic form is characterized by development of lung fibrosis[2]. The disease does not necessarily progress form a cute to the chronic form[1]. PFL is caused these type III reaction and patients have precipitating antibodies to pigeon antigens, and these gave rise to an Arthur type kin-test reaction although detection of these antibodies against pigeon antigens in asymptomatic pigeon fanciers a poses a problem for the immune complex model of disease[3]. Individuals with PFL have antibodies specific for a range of pigeon antigens including proteins, glycoproteins and polysaccharides. The lymphocytic infiltrate in PFL consists mainly of T cells. In previous studies it was show that in patients who were isolated from sources of antigen for 3 weeks the number of lymphocytes were reduced in the alveoli suggesting that lymphocyte numbers are maintained by antigen exposure[3]. Fancier's with PFL have significantly higher titer of anti-mucin IgG1 than asymptomatic antibodies-positive individuals. Anti-mucin IgG1 in immune complexes may activate FcY receptor on neutrophils/macrophages better than IgG2 which is found in very high levels in asymptomatic fancier's[1].

Immunotherapy and allergen avoidance are the only treatments that may affect the natural course of allergic diseases[4]. In spite of the fact that allergen avoidance has been crucial for treatment of allergic symptoms, pharmacotherapy with injectable epinephrine, oral antihistamine for the management of the anaphylaxis is recommended[5]. Allergen avoidance to pigeon is the best therapy for Pigeon fancier's lung (PFL). However, allergen avoidance is not possible because of occupational exposure, immunotherapy may be the treatment of choice in cases of pigeon allergy. Some studies indicate that exposure to animals at homes during the 1st year of life and early exposure to farm animals leads to a protective effect against animal induced allergy and asthma, by development of immune response by immunoglobulin (IgG) directed against animal dander[5]. Immunotherapy is specific to the antigen administered. Recently, Scientific studies reported that immunotherapy acts by modifying T-cell responses either by immune deviation (increase in T_{H0}/T_{H1}), T-cell energy (decrease in T_{H2}/T_{H0}), or more likely both. In addition, systemic and local increases in CD8+ cells have been observed. Immunotherapy also decreases inflammatory cell recruitment and activation and mediator secretion[4]. As reviewed, Pigeon breeders' disease is form of hypersensitivity pneumonia commonly affects adults, described infrequently in children. Immune complex (type III) reactions may play important role in PFL[6,7]. Type III reactions involve tissue injury by immune complex[8]. This response occurs when an antigen reacts in the tissue spaces with potentially precipitating antibodies forming micro precipitating in and around small vessels, causing secondary damage to cells. When antigen levels increase, soluble immune complexes are

found and more deposited in the endothelial lining of blood vessel walls, fixing complement and causing local inflammation[8]. Serum sickness, which develops 9-10 days following the administration of heterologous therapeutic sera, such as anti thymocyte globulins[9], is the most typical example of type III hypersensitivity reactions. Serum sickness has also been suspected to develop following the administration of low molecular weight drugs. However, in most instances, no circulating or deposited immune complex can be detected, hence the preferred term 'serum sickness-like disease'[10].

It was detected that pigeon fancier's who smoke show depressed antibody responses to pigeon antigens compared with non-smokers[6].

It is reported that therapy by corticosteroids medication can inhibit the antigen induced response of lymphocytes from patients with pigeon breeder's lung, and that false negative diagnostic LT tests for PBL may occur as a result[11].

It was reported that early treatment with a corticosteroids medication leads to complete reversal in acute and sub-acute hypersensitivity pneumonia[6]. Chronic hypersensitivity pneumonia may progress to irreversible lung damage in spite of treatment and avoidance of the offending antigen[5].

Recant study reported that inhalation challenge with pigeon serum and pigeon dropping extract (PDE) elicits a hypersensitivity reaction in patients with bird-related hypersensitivity pneumonitis (BRHP), but the antigenic components in these materials have yet to be fully elucidated[12].

Neutrophils play an important role in defence against extra cellular pathogens by means of phagocytosis clearing these bacteria from blood stream and infected tissues. Patients with deficiencies in number or function of phagocytes are prone to develop pyogenic infections that might be life threatening[13]. Neutrophils are produced in vast numbers (1-2x 10^{11} per day) in the bone marrow.

Hypothesis and aim:

The hypothesis to be tested in this study is that immune complex from symptomatic pigeon fanciers are better at inducing inflammatory responses in macrophages than immune complex from asymptomatic fanciers

The aim of this study was to set up system measure macrophage activation by immune complexes. By

A- Determination of the optimal time for macrophage activation

B- Determination of the best conditions for cytokine production with a positive control stimulate (bacterial lipopolysaccharide LPS)

C- Determination of the best method of adding immune complex to the cells.

D- Determination of the best concentration of immune complexes to add to the cells.

MATERIALS AND METHODS

Cell lines:

The human macrophage-monocytic cell line U937 and Thp-1 were stored in liquid nitrogen, both cell lines were supplied by ATCC .

The cells were thawed rapidly and placed into a 10 ml centrifuge tube. 10 ml of RPM1 was added and the cells were spun at 2000 r.p.m for 10 minutes. The supernatant was removed and cells were washed twice in RPM1. The cells were then maintained in RPM1 1640 (Gibco Life Technologies Ltd, paisley, UK) supplemented with fetal calf serum (10%), glutamine, penicillin, and streptomycin in standard concentrations (RF10).

Patients:

This study was conducted in Northumbria university laboratory on 80 male volunteers from Newcastle, the patients were classified in to four groups according to their clinical status and the presence or absence of precipitating antibodies. The groups as the follows:

The patients have been classified in to four groups according to their clinical status and the presence or absence of precipitating antibodies. The groups as the follows:

Group A, symptomatic with precipitating antibodies.

Group B, asymptomatic with precipitating antibodies.

Group C, symptomatic without precipitating antibodies.

Group D, asymptomatic without precipitating antibodies.

(Table 1) details of 80 pigeon fanciers in study

Details	Group A	Group B	Group C	Group D
Number of subjects	12	28	10	30
Mean age in years	52	53	54	51
Percentage of using the mask	80%	28%	50%	20%
Number of pigeon kept	61	67	75	72
Number of Years kept pigeon	24	28	27	20
Hours contact per week	15	20	20	25
Percentage of current smokers	16%	10%	30%	33%

Antigens: a number of different antigens were used to make up the immune complex (IC) mucin, fresh pigeon dropping (PDF), and old pigeon droppings (PDO). Prior the antigens for I.C the antigens were tested by ELISA to show antibodies response to this antigens. Mucin was diluted in coating buffer 1/1000. PDF and ODF at 1/2000 then 100µl was added in to each well of a 96 well microplate. The plates were kept overnight in incubator at 37C˚. An-

tigens was removed and 100ul of 3% bovine serum albumin in PBS was added to each well and incubated for 1 hour. Plates were washed in PBS-Tween and then sera were doubling diluted across the plate staying from 1/500, plates were incubated for 90 minutes, washed and then 100µl of antihuman IgG peroxidase at 1/2000 was added to each well and incubated for 1 hour, plates were washed and developed with OPD. The reaction was stopped with 12.5% H_2SO_4 and plates were read at wavelength 490 nm.

Cell activation by (PMA): 200µl of cells were plated out in the wells of flat bottom plate at $0.5x10^6$/ml in RF10 containing 100µg/ml phorbolmysteric acetate (PMA) which induces monocytes to differentiate in to macrophage, cells were incubated at 37°C in 5% CO2 for 24, 48 or 72 hours, and then the supernatant were collected to determine the best time for cell maturation. In series of separate experiments 200µl cells at $0.5x10^6$ /ml in RF10 without PMA. Cells were incubated at 37°C in 5% CO2 for 24, 48, or 72 hours and the supernatants were collected and screened by ELISA technique for TNFα.

Lipopolysaccharide (LPS) activation of U937 and Thp1:
Both U937 and Thp1 cells at 0.5x106/ml were incubated with PMA at 100 µg /ml for 72 hours. The supernatant was removed and 200uL of LPS in RF10/ml, 5µg /ml, 2µg /ml, 1g/ml, and o.5 µg /ml was added to the cells. Cells were incubated in LPS for 24 and 48 hours. The supernatant were collected and TNFα was detected by ELISA. U 937 and Thp1 at 0.5x106 /ml were activated with a mixture of LPS at 5µg /ml and PMA at 100g/ml and plates were incubated for 24, 48, and 72 hours. Supernatant were collected to detect concentration of TNFα production.

ELISA technique to measure TNFα:
ELISA plate were coated overnight with 1µg/ml of monoclonal anti-human TNFα. The Ag was removed and wells were blocked with 100µL phosphate buffered saline (PBS) containing 3% Bovine albumin for 30 minutes; subsequently, washed three times with PBS and then washed once with PBS/Tween followed by washing three times with distilled water . TNFα standard and macrophages supernatant were added to wells and incubated for 90 minutes followed by washing as previous. TNFα biotin antibody (second antibody) was added to each well for 1 hour and after washing 100µl of 1/1000 extravidin (conjugate) was added for 30 min. Following the final washes the substrate o-phylenediamin di-hydrochloride (OPD sigma) was added and enzymatic reaction allowed to produce for 30 minutes. The reaction was stopped by adding 25 µl of 12.5% H2SO4 and the absorbance was measured at 490 nm.

RESULTS

Activation of macrophage cell lines with PMA and LPS:
U937 and Thp-1 cells at $0.5x10^6$/ml were cultured with PMA at 100ng/ml for 24, 48, 72 hours. Supernatant were removed and then LPS at 5µg/ml were added to the cells for 24,48, 72 hours. Supernatant were collected for TNFα.

(Table 2) TNFα concentration in different experimental

No of column	Incubation time (all the cells at $0.5x10^6$)	TNFα concentration
1	24h PMA + 24h LPS	1.5 ng/ml
2	24h PMA + 48h LPS	1.48 ng/ml
3	24h PMA + 72h LPS	1.22 ng/ml
4	48h PMA + 24h LPS	1.5 ng/ml
5	48h PMA + 48h LPS	1.6 ng/ml
6	48h PMA + 72h LPS	2.1 ng/ml
7	72h PMA + 24h LPS	4 ng/ml
8	72h PMA + 48h LPS	3.5 ng/ml

TNFα was detected in U 937 cells after activation of cells with PMA and subsequent stimulate with LPS. This shows that U937 cells must be cultured with PMA before they will produce cytokines. The concentration of TNFα produced by U937 cells with LPS after with PMA is showed in table 2. The highest level of TNFα produced by U937 was after incubated with PMA for 72 hours and LPS for 24 hours. TNFα was never detected after PMA and LPS activation by monocyte/macrophage cell line Thp1. Macrophage activation by immune complexes.

U937 cells at 0.5x106/ml were cultured with PMA at 100ng/ml for 72 hours. The supernatant was removed and then different concentration of soluble immune complexes were added to the cells. The make up the immune complex is shown in (table 3,4,5) thus 9 different concentrations of immune complex for each added to cells.

The highest level of TNFα produced by the U937 cells immune complexes. Macrophage cytokine never activated by immune complex content mucin and sera.

(Table 3) The optimal concentration of immune complexes for macrophage activation by using soluble immune complexes (PDF + sera)

No	Ratio Ag : sera	TNFα at 24 hours	TNFα at 48 hours
1	1:10	17 ng/ml	21.5 ng/ml
2	1:1	31 ng/ml	27.2 ng/ml
3	10:1	30 ng/ml	28.5 ng/ml
4	1/10 dilute of No 1	22.1 ng/ml	17.5 ng/ml
5	1/10 dilute of No 2	17.8 ng/ml	15 ng/ml
6	1/10 dilute of No 3	17 ng/ml	17.1 ng/ml
7	1/100 dilute of No 1	5.5 ng/ml	4.7 ng/ml
8	1/100 dilute of No 2	6.3 ng/ml	6 ng/ml
9	1/100 dilute of No 3	11 ng/ml	11.2 ng/ml

(Table 4) The optimal concentration of immune complexes for macrophage activation by using soluble immune complexes (PDO + sera)

No	Ratio Ag : sera	TNFα at 24 hours	TNFα at 48 hours
1	1:10	5.9 ng/ml	6 ng/ml
2	1:1	15.2 ng/ml	16.2 ng/ml
3	10:1	15.4 ng/ml	15.2 ng/ml
4	1/10 dilute of No 1	2.8 ng/ml	1 ng/ml
5	1/10 dilute of No 2	5.1 ng/ml	1.1 ng/ml
6	1/10 dilute of No 3	4.1ng/ml	5.7 ng/ml
7	1/100 dilute of No 1	0 ng/ml	0 ng/ml
8	1/100 dilute of No 2	0 ng/ml	0 ng/ml
9	1/100 dilute of No 3	0 ng/ml	0 ng/ml

(Table 5) The optimal concentration of immune complexes for macrophage activation by using soluble immune complexes (mucin + sera)

No	Ratio Ag : sera	TNFα at 24 hours	TNFα at 48 hours
1	1:10	0 ng/ml	0 ng/ml
2	1:1	0 ng/ml	0 ng/ml
3	10:1	0 ng/ml	0 ng/ml
4	1/10 dilute of No 1	0 ng/ml	0 ng/ml
5	1/10 dilute of No 2	0 ng/ml	0 ng/ml
6	1/10 dilute of No 3	0 ng/ml	0 ng/ml
7	1/100 dilute of No 1	0 ng/ml	0 ng/ml
8	1/100 dilute of No 2	0 ng/ml	0 ng/ml
9	1/100 dilute of No 3	0 ng/ml	0 ng/ml

Macrophage activation by different immune complex:

U937 cell line at $0.5x10^6$/ml were cultured with PMA at 72 hours. The supernatant was removed and then 10ul if different immune complex that were prepared as described in the material and methods with different sera and PDF at a ratio 1:1 were incubated for 24 hours with the cells. The supernatant were collected and tested for TNFα production. The TNFα production for each individual sera of all groups (A, B, C and D) (table 6).

(Table 6) The median of TNFα concentration in each group.

Groups	Minimum	Maximum	Median	25% (Q1)	75% (Q1)
A	16.2	24%	18.2	17.2	21.67
B	13.5	26	17.5	16.625	19.150
C	13.5	22.1	18.3	16.375	19.750
D	1.5	23.5	18.2	16.500	19.125

DISCUSSION

The basic pathogenesis of pigeon fancier's lungs (PFL) starts with the production of antibodies, which interact with inhaled mucin antigens, forming immune complexes in the patient's alveoli. However, large amounts of anti mucin IgG are found in asymptomatic patients as well as symptomatic patients thus it is difficult to understand why some people present with symptoms and other do not[3]. In contrast symptomatic patients have higher anti mucin IgG1 titres than asymptomatic and it has been suggested that as IgG1 better at activation this may be the reason of disease developed[3]. Similar results have been reported in mollusc shell dust extrinsic allergic alveolitis where high levels of specific IgG1 been implicated in the development of disease[14]. Antibodies to pigeon mucin are present at high titres in the serum of symptomatic (Group A individuals), and asymptomatic (Group B individuals) and at low levels in symptomatic individuals (Group C) and asymptomatic individual (Group D)[3]. In this study we investigated if there were difference in the ability of immune complex from all of these patient groups to activate macrophages. The major pathology of acute type PFL is probably due to immune complex deposition, and this may be exacerbated by increased vascular permeability caused by mast cell activation via FcY RIII[8]. The deposited immune complex trigger neutrophils through Fc YRs to discharge their granule contents with consequent damage to the surrounding endothelium and basement membranes[8]. In this study we have set up an assay to measure the ability of immune complexes to activity macrophages. It would be more realistic to use neutrophils but this is difficult as we would fresh blood for each and every experiment and there is no established neutrophil cell lines as neutrophils have similar receptors on their surface as macrophage and have the same ability to produce cytokines in response to immune complex we have used two macrophage cell lines to study the effects of immune complex. There were stimulated with PMA and then immune complexes generated with Ag and patient sera and the read out of activation was the production of cytokine tumour necrosis factor α (TNFα). In this study the two macrophage cell lines Thp1 and U937 were used. Both monocytic cell line were cultured and PMA was added to stimulate the monocytes to terminal differentiation to macrophages. Activation of the human monoblastic leukaemia cell line U937 by phorbol 12-myristate (PMA) increases

the expression of CD14/CD86, and cytokine production[15]. Production of CD14 mRNA is dependent on the activation of protein kinase C[16]. Bacterial endotoxin (Lipopolysaccharide, LPS) is a complex glycolipid composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A. LPS is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation[17]. LPS activates monocytes and macrophages to produce pro inflammatory cytokines such as TNFα, interleukin (IL)-, IL6, IL8, and IL-12. Macrophages also secrete, in response to LPS, a wide variety of other biological mediators including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide[17].

CD14, a 55-kDa glycoprotein, is an essential component of the LPS receptor complex CD14 is present in soluble form in blood or a glycosylphosphatidylinositol-linked form on myeloid lineage cells. Enterobacterial LPS firs binds to a serum LPS binding protein (LBP), which in turn transfers a LPS monomer form the bacteria cell wall to membrane-bound CD14 on monocytes and myeloid cells, subsequently causing LPS responses[18] CD14-negative cells such as endothelial cells and epithelial cells also respond to LPS because soluble CD14 can substitute for membrane-bound CD14. Besides LPS, CD14 is also required for the recognition of other bacterial products including peptidoglycan, lipoteichoic acid, and lipoarbinomannan[19].

Immune complexes were generated with mucin, fresh pigeon dropping (PDF), old pigeon droppings (PDO), and patient sera. After the assay had set up immune complexes were added to macrophages. Both solid phase and soluble immune complexes were generated, soluble immune complex were easy to prepare and add to cell, whilst solid phase immune complex were harder to prepare but well more closely that of the immune complexes formed in the alveolar in PFL. The results showed that immune complexes with sera and PDF and PDO activated U937 cells activity production of TNFα with immune complexes generated with mucin and sera did not. This was unexplained as it was that immune complexes and mucin would activate the cells. Reason for this absent of activation may be because the antigen molecular size, charge, structure, amount, and valence influence the type of immune complexes that are formed and this may not be good at macrophage activation. The size of immune complexes generated with mucin be very large compared with pigeon dropping material which will contain may smaller molecules and these smaller complexes may be better at macrophage activation. We also showed the PDF was better as activator of macrophages than PDO, it may be that mucin in the droppings is acting as an activate antigen to make immune complexes.

CONCLUSION

Antibodies to pigeon antigens are present in the serum of symptomatic (Group A individuals) and asymptomatic (Group B individuals) antibody positive fanciers and symptomatic (Group c) and asymptomatic antibody negative individuals (group D). The first part of this study was to set up a system that measured the activation of monocytic cells lines by both PMA and LPS and was successful. The observation that there was no effect of LPS on the U937 cell line without prior stimulation with PMA can be explained by the fact that PMA increases the expression of CD14 on the surface of the cells. LPS activates monocytes and macrophages to produce pro-inflammatory cytokines such as TNFα after stimulation with PMA. The second part of this study was to determine if immune complexes could induce the production of pro-inflammatory cytokines by the cells and this was also successful. Immune complexes with sera and PDF and PDO activated U937 cells leading to the production of TNFα, while immune complex generated with mucin and sera did not. All immune complexes with PDF and different sera groups showed similar effects for macrophages activation. Thus suggesting that there is no difference in the ability of these immune complexes to induce pro-inflammatory response.

Finally clinicians should be aware of the pigeon breeder's disease progression, irreversible lung damage and specific allergen immunotherapy with pigeon antigen is may be effective and lifesaving.

Further study:

It may be interesting to estimate if macrophage can be activated by immune complex with mucin extracted from both PDF and PDO by (Cs CL) as mucin in these natural antigens will be different from that in the pigeon intestinal mucin tested here. It may also be useful to determine the minimal antibody concentration that have effects on macrophage activation by preparing different titrations of patient sera to produce immune complexes with both PDF and PDO.

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