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LECTIN HISTOCHEMISTRY OF CHOLESTEROL CLEFT GRANULOMAS IN NON-SPECIFIC INTERSTITIAL PNEUMONIA (NSIP)

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ABSTRACT

Background: Cholesterol cleft granulomas with clusters of giant cells were noted to be a common feature of non-specific interstitial pneumonia (NSIP).

Objective: This study aimed to define the cell populations involved in the granulomas.

Methods: The granulomas of 16 patients with cryptogenic fibrosing alveolitis (five cases with the histological features of NSIP, five with those of UIP and six cases of respiratory bronchiolitis) were examined histologically and by the use of immuno- and lectin histochemical markers.

Results: Granulomas were discrete, compact and present only in alveolar spaces. The adjacent interstitium usually showed fibrous thickening although granulomas were absent. The granulomas contained central clefts surrounded by mononuclear and multinucleated giant cells, both of which were CD68 positive. The cells outside the granulomas and those lining the adjacent alveolar walls were AE1/AE3 and CAM5.2 positive and CD68 negative. The application of an extended lectin panel demonstrated restricted glycoprofiles for multinucleated cells, alveolar macrophages and alveolar lining cells. The glycoprofiles of the first two were similar to each other, but were different from the third.

Conclusion: The mononuclear and multinucleated cells of cholesterol cleft granulomas are derived from the macrophage-mononuclear cell lineage and express glycoproteins with a high mannose content. The alveolar lining cells are type II pneumocytes which do not contribute to the granuloma cell population.

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INTRODUCTION

The pathological classification of idiopathic pulmonary fibrosis (IPF) - also known as cryptogenic fibrosing alveolitis (CFA) - has been a matter of difficulty and controversy for histopathologists.[1-5] A recent classification by Katzenstein and Myers[6] includes usual interstitial pneumonia (UIP),

desquamative interstitial pneumonia (DIP)/respiratory bronchiolitis interstitial lung disease (RBILD), acute interstitial pneumonia (AIP, Hamman-Rich disease) and non-specific interstitial pneumonia (NSIP). This last was defined by Katzenstein and Fiorelli[7] as an idiopathic interstitial pneumonia with a pathological pattern distinct from UIP, DIP and AIP, although cases were first recognised because they could not be included in the first three categories.

In a systematic evaluation of the histopathological features of a series of IPF we noted that half the cases of NSIP contained large numbers of discrete cholesterol cleft granulomas. Examination of the available literature revealed few references to these entities in NSIP. In two of the original series (n=64) of Katzenstein and Fiorelli,[7] loosely formed granulomas, one containing cholesterol clefts, were described. In 12 cases described by Cottin et al[8] six had small, loosely formed granulomas consisting of a cluster of epithelioid cells, giant cells and cholesterol clefts. Finally, Katzenstein and Myers[6] refer to rare, focal, poorly formed, non-necrotising granulomas. We were, therefore, prompted to examine the granulomas in our series in detail and to consider their pathogenesis and possible significance.

MATERIAL AND METHODS

Nine formalin-fixed, paraffin-embedded blocks from six cases of non-specific interstitial pneumonia (NSIP) were obtained from the histopathology archive of the Wythenshawe Hospital Manchester. Immunohistochemistry and lectin histochemistry were performed.

Immunohistochemistry

Paraffin-embedded sections (5 μm), cut as near serially as possible, were dewaxed, blocked for endogenous peroxidase, rehydrated and immunostained with the monoclonal antibodies AE1/AE3, CAM5.2, anti-CD68 and anti-mast cell tryptase as detailed in Table I. Sections were pre-treated with 0.03% (w/v) trypsin (type II, porcine, Sigma) stained directly with anti-CD68 and indirectly with AE1/AE3, CAM5.2 and anti-tryptase using the avidin-biotin method with 3-3-diaminobenzidine tetrahydrochloride as the substrate and Mayer's haematoxylin as the counterstain.

Lectin histochemistry

Sections (5 μm) were again cut as near serially as possible, dewaxed, blocked for endogenous peroxidase, rehydrated and stained with a panel of biotinylated lectins, after trypsinisation, according to the method of Jones et al [9] and Barkhordari et al.[10] Briefly, the biotinylated lectins were applied at a concentration of 10 μg/ml (MAA lectin at 20 μg/mL) at room temperature for 30 minutes. After washing, sections were treated with avidin-conjugated peroxidase at 5 μg/mL in 0.125M TBS, pH 7.6, containing 0.374M sodium chloride, for one hour. Subsequently, 3,3-diaminobenzidine tetrahydrochloride was used as the substrate and the sections were routinely counterstained with methyl green. Details of the origins and specificities of the 27 lectins used are given in Table II. The biotinylated lectins were obtained from Sigma apart from SNA and MAA, which were from Boehringer Mannheim, AAA from EY Laboratories Inc. and GNA, NPA and HHA from Vector Laboratories. Neuraminidase pre-treatment was applied to remove terminal sialyl residues from oligosaccharide chains thereby allowing exposure of sub-terminal glycan sequences to the lectins. This pre-treatment was carried out after trypsin digestion by incubating the sections at 37°C in a solution of neuraminidase (type VI, from *Clostridium perfringens*, Sigma) at 0.1 units/mL in 0.01M sodium acetate buffer, pH 5.5, containing 1% (w/v) calcium chloride, for one hour and repeating once more with fresh enzyme. Controls included negative controls with buffer substitution for lectin, competing sugars and, for SNA and MAA, the neuraminidase pre-treatment. Staining intensity was ranked using (-) for none, (+) for just detectable, weak staining, (++) for moderate clear staining, (+++) for strong staining and (++++) for intense staining.

RESULTS

Immunohistochemistry

Half of the cases studied exhibited the presence of numerous dispersed cholesterol cleft granulomas (Figure 1). These were discrete, compact, uniform in size and present mostly in areas of alveolar wall thickening. The granulomas were universally present in alveolar spaces. They were never observed within alveolar walls nor in relationship to blood vessels. Typically the granulomas contained central clefts (mean length 52.6 μm, range 18.0 109.3 μm) surrounded by mono- and multinucleated cells which were CD68 positive, whereas the cells outside the granulomas and lining the alveolar walls were negative with this antibody (Figure 2). In contrast these latter cells were both AE1/AE3 and CAM5.2 positive (Figure 3). Elsewhere, in alveolar spaces not containing granulomas, there were occasional small populations of alveolar macrophages also showing CD68 positivity.

Anti-mast cell tryptase positive cells were present in the alveolar wall interstitium, frequently close to the lining cells, but never within the alveolar space nor in intimate relationship to the granulomas.

Lectin histochemistry

Of the 27 lectins tested, 19 bound to mono- and multinucleated cells either in the granulomas or in non-granulomatous mononuclear cell populations in the alveolar spaces. Ten lectins specifically, but weakly, stained multinucleated cells (GNA, NPA, PSA, LCA, SNA, MAA, HPA, LEA, PAA and sWGA) whereas six stained them strongly (HHA,

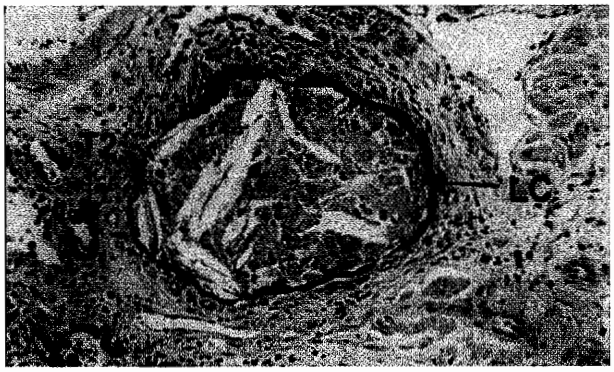

Fig 1. Strong staining of type II pneumocytes (T2) and cholesterol cleft granuloma lining cells (LC) with CAM5.2.

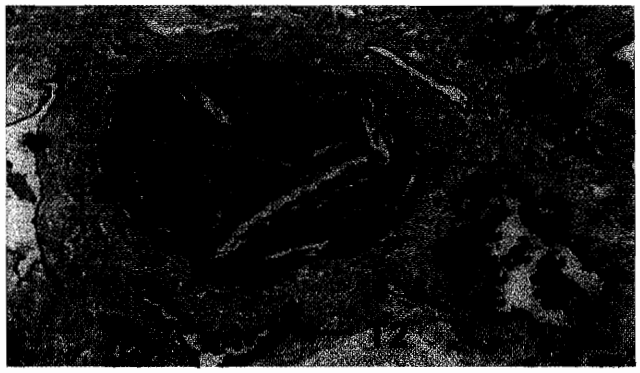

Fig 3. LEA staining of giant cells and cholesterol cleft granuloma lining cells.

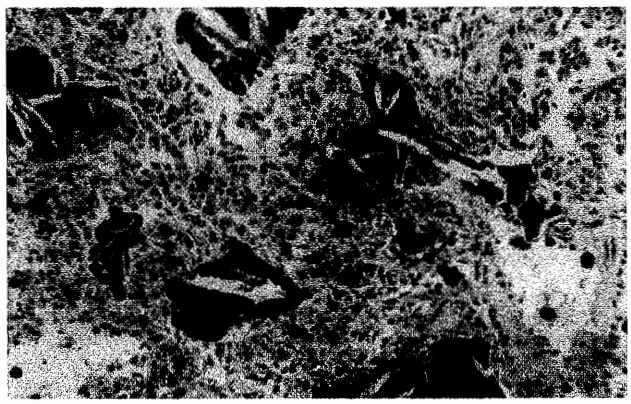

Fig 2. Strong staining of mono- and multinucleated giant cells with anti-CD68.

Fig 4. HPA stained the type II pneumocytes lining the cholesterol cleft granulomas and endothelial cells.

ConA, ECA, MPA, WFA and PTL-I). The multinucleated giant cells in the granulomas had a more restricted staining pattern than the intra-alveolar macrophages, with lesser levels of staining with all lectin groups except for HPA of group 5 and PTL-I of group 7. The intra-alveolar macrophages were predominantly membrane-positive with e-PHA, l-PHA and WFA in NSIP, both with and without granulomas: this membrane staining was not found in normal lung. ECA, as

Table I. Details of the antibodies used.

Antibody	Dilution	Reactivity	Control Tissue	Source
AE1/AE3	1:50	Human epidermal keratin	Skin	Dako
CAM5.2	Neat	Cytokeratins 8,18,19	Tonsil	BD Biosciences
Anti CD68 (EBM11)	Neat	Macrophages	Tonsil	Dako
Anti-mast cell tryptase (AA1)	1:100	Mast cells	Tonsil	Novocastra Laboratories Ltd.

Table II. The sources and specificities of the lectins used.

Group 1	**Mannose in various subsets of N-glycans**	
GNA	*Galanthus nivalis* /snowdrop	Non-reducing terminal αDMan, especially Manα1,3 Man linkage
NPA	*Narcissus pseudonarcissus* /daffodil	Non-reducing terminal & internal αDMan, especially Manα1,6 Man linkage
HHA	*Hippeastrum hybrid* /amaryllis	Non-reducing terminal & internal α (DMan, in α1,3, α1,6 and α1,2 linkages
Con A	*Canavalia ensiformis* /Jack bean	Terminal αD Man and αDGlc, plus 1,2-linked internal αD Man in bi-antennary complex N-glycans and meso-inositol
PSA	*Pisum sativum* /garden pea	αDMan in non-bisected bi/tri-antennary, complex N-glycans with core fucosylation
LCA	*Lens culinaris/lentil*	Similar to PSA, 'prefers' terminal GlcNAc
e-PHA	*Phaseolus vulgaris* haemagglutinin/kidney bean	Bi/tri-antennary,
l-PHA	*Phaseolus vulgaris* leukoagglutinin/kidney bean	GlcNAcβ1,2Man in tri/tetra-antennary, plex N-glycans, unless there is a proximate sialyl residue
Group 2	**α-L-Fucosyl termini**	
UEA-I	*Ulex europaeus/gorse*	α-L-Fucosyl termini especially L-Fucα1,2Galβ1,4GlcNAcβ1-
LTA	*Tetragonolobus purpureus* /winged or asparagus pea	α-L-Fucosyl termini (especially where clustered)
AAA	*Anguilla anguilla* /freshwater eel	α-L-Fucosyl termini and fucosylated type I chains
Group 3	**α-N-Acetylneuraminyl (and other sialyl) termini**	
SNA	*Sambucus nigra* /elder tree bark	NeuNAcα2,6Gal/GalNAcβ1-
MAA	*Maackia amurensis*	NeuNAcα2,3Galβ1-
Group 4	**Variously branched glycans with β-galactosyl termini**	
ECA	*Erythrina cristagalli* /cocks comb coral tree	Galβ1, 4GlcNAcβ1- (and Galα1, 3Galβ1, 4GlcNAcβ1-)
AHA	*Arachis hypogaea* /peanut	Galβ1,3GalNAcα1- > Galβ1, 4GlcNAcβ1-
Group 5	**α-2-Deoxy,2-acetamido-galactose, in various linkages**	
DBA	*Dolichos biflorus* /horse gram	GalNAcα1,3 (LFucα1,2) Galβ1, 3/4GlcNAcβ1-
VVA	*Vicia villosa/hairy vetch*	GalNAcα1,3 Galβ1- > GalNAcα1, 6 Gal-
MPA	*Maclura pomifera/osage orange*	Galβ1,
PTL-II	*Psophocarpus tetragonolobus* /winged bean	Galβ1, 3GalNAcα1- > GalNAcβ1, 3GlcNAc > NAcLac
SBA	*Glycine max/soybean*	Terminal GalNAcα1-> Galα1- or GalNAcβ1- in longer chains
HPA	*Helix pomatia/edible snail*	GalNAcα1-
WFA	*Wisteria floribunda* /Japanese wisteria	GalNAcα1, 6Galβ1- > GalNAcα1, 3Galβ1-

Group 6	Linear and branched oligomers of N-acetyl lactosamine: di-N-acetyl chitobiosyl sequences	
LEA	*Lycopersicon esculentum* /tomato	$(-4GlcNAc\beta1-)_{2-4}$
PAA	*Phytolacca americana* /pokeweed root	$(-4GlcNAc\beta1-)_{n}$
sWGA	*Triticum vulgaris*	$(-3Gal\beta1, 4GlcNAc\beta1-)_{n}$ and
	/succinylated wheat germ	$(-4GlcNAc\beta1-)_{n}$
Group 7	**Terminal α-galactose**	
$BSA\text{-}IB_4$	*Griffonia simplicifolia*	Galα1,3Gal-
PTL-I	*Psophocarpus tetragonolobus* /winged bean	Galα1-

well as the strong membrane staining, also showed quite strong granular cytoplasmic staining of the alveolar macrophages when granulomas were present. AHA stained a few alveolar macrophages around the granulomas, but, after removal of sialyl residues by neuraminidase pre-treatment, more cells reacted. The lectins LEA, PAA and sWGA of group 6 showed a change in pattern of staining from mostly membranous in NSIP without granulomas to cytoplasmic when granulomas were present. The alveolar wall lining cells were specifically identified by twelve lectins: HHA, ConA, SNA, MAA, AHA, MPA, HPA,WFA, LEA, PAA, sWGA and PTL-I (Figures 3 and 4). The mast cells of the alveolar walls had a limited reaction with the lectins used with the strongest binding by PSA, LCA and ECA (results not shown).

DISCUSSION

The histopathological features of NSIP include interstitial inflammation and/or fibrosis in varying proportions from a pure chronic inflammatory cell infiltrate to fibrosis without a cellular infiltrate. The most characteristic feature of NSIP is that the lesions are temporally uniform,[8] in distinction to the temporal heterogeneity of UIP. NSIP was originally defined by the exclusion of cases that could not be placed in the other well-defined categories of IPF. There does, however, appear to be some distinguishing features from UIP and the prognosis is apparently better. Underlying connective tissue disease, inhalation of organic dust and slowly resolving acute lung injury have all been implicated in the aetiopathogenesis of NSIP.[11]

The presence of cholesterol cleft granulomas was a distinctive feature of half of our cases of NSIP. The granulomas were well defined with central clefts surrounded by macrophages and macrophage polykaryons. The granulomas were compact and confined to spaces lined clearly and separately from the cells of the granuloma, by a complete single layer of type II pneumocytes. Outside this, the interstitium was fibrotic and contained a population of mast cells demonstrable by anti-tryptase immunostaining. Immunopositive material had apparently diffused outside the cell boundaries suggesting tryptase release. Mast cells were never seen within the granulomas although an occasional cell was present in the type II pneumocyte lining and in the space between this lining and the cells of the granuloma.

Granulomatous inflammation to cholesterol crystal clefts has been described in a number of pulmonary disorders including idiopathic cholesterol pneumonitis[12] and as a secondary phenomenon in bronchiectasis, chronic lung abscess and tuberculosis.[13-15] The source of the cholesterol is likely to be pulmonary surfactant. In an electron microscopical study of lung tissue from heavy cigarette smokers, Corrin and Soliman[16] found cholesterol crystal clefts in the cytoplasm of type II pneumocytes. Lipid extracts of bovine pulmonary surfactant contained 3% neutral lipid mainly as cholesterol and diacylglycerol and 97% phospholipid.[17]

The giant cells commonly seen in granulomas are considered to be macrophage polykaryons formed by the fusion of alveolar macrophages attached to the same endocytic material.[18] Molecules involved in the fusion of macrophages included originally a lymphocyte derived macrophage fusion factor[19] and latterly interleukins (IL) 4 and 13.[20-21] In addition, these interleukins are produced and stored by mast cells[22-24] which regulate fibroblast proliferation in vitro by heterotypic cell-cell contact and secretion of IL-4.[25] In an immunohistochemical study of idiopathic pulmonary fibrosis IL-4 positive mast cells were observed in greater numbers in advanced versus early lesions.[26]

The chemical nature of the macrophage membrane receptor for the fusion factor was suggested by the findings, *in vitro*, that pre-treatment of macrophages with β-mannosidase, or incubation in the presence of β-D-mannose completely inhibited giant cell formation.[27] Further, in

macrophage fusion systems producing multi-nucleated cells, mannose receptors are up-regulated specifically by IL-4 and are present and concentrated at macrophage fusion interfaces.[28] An identical effect is produced by IL-13.[21]

Whilst exogenous cytokines are influential in granuloma formation and activity, endogenous production of cytokines also occurs. Human non-caseating pulmonary tuberculous granulomas contain CD68 positive macrophage-like cells which produce mRNA for TNF-α, IFN-γ and IL-4 and these are likely to have functional significance. [29] Currently there are no available data on cytokine elaboration by pulmonary cholesterol cleft granulomas.

This immunohistochemical study confirmed that the intra-alveolar cell populations in NSIP were composed predominantly of CD68 positive cells. It showed that the giant cells were also CD68 positive, but were cytokeratin negative. In contrast, the cells lining the alveolar spaces containing granulomas were cytokeratin positive (AE1/AE3 and CAM5.2), indicating that these were type II pneumocytes[30] and showing that these cells had completely replaced type I cells at these loci.

These immunohistological findings were confirmed by the results of the glycoprofiling studies. These generated detailed information about the nature of the cellular glycoconjugates. In addition, it is known that macrophages and macrophage polykaryons have distinctive glycoprofiles and that pneumocytes types I and II can be distinguished by their profiles.[10,31-32] The results suggest that the mononuclear and multinucleated cells of cholesterol cleft granulomas are derived from the macrophage-mononuclear cell lineage and express high mannose N-glycans which we would suggest have a role in macrophage fusion to the known surface mannose receptors.[33] After fusion there is down regulation of components of the glycoprofile which appear to contribute to the fusion process. In addition, other elements of the glycoprofile are diminished in multinucleated versus alveolar macrophages indicating that glycan synthesis is reduced. The alveolar macrophages had slightly different glycoprofiles from those found in normal lung (results not shown). NSIP macrophages demonstrated the presence of terminal β-galactosyl and more 2-deoxy,2-acetamidogalactosyl residues. The cells lining the alveolar spaces demonstrated the complex glycotype which distinguishes type II from type I pneumocytes.[10] In addition, staining of type II pneumocytes by LEA, PAA, sWGA, AHA, HPA, WFA and ECA, which were negative in normal lung, demonstrated the presence of high levels of (-4GlcNAcβ1)$_n$, (-4GlcNAcβ1-)$_{2-4}$ and/or (Galβ1,4GlcNAcβ1)$_n$, Galβ1, 3GalNAcα1-, Galβ1,4GlcNAcβ1-, terminal GalNAcα1- and GalNAcα1, 6Galα1- and Galα1 sequences. The mast cells in these cases demonstrated a wider range of lectin-binding glycans than was found in normal lung tissue. The high mannose N-glycans (HHA) and glycans with β-galactosyl termini (ECA) or N-acetylgalactosamine (MPA) or N-acetyllactosamine (LEA) were detected in mast cells in NSIP but not in normal lung.

In summary, our observations indicate that cholesterol cleft granulomas occur with high frequency in NSIP, are present within alveolar spaces which are lined exclusively by type II pneumocytes and that external to this the interstitium is fibrotic with of mast cells. It is our speculation that these are linked in a pathogenetic mechanism related to the progression of NSIP. The results also suggest that the mononuclear and multinucleated cells of cholesterol cleft granulomas are derived from the macrophage-mononuclear cell lineage. The alveolar lining cells are type II pneumocytes which do not contribute to the granuloma cell population.

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