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Immunohistological study of human lungs by immunoperoxidase technique

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SUMMARY An unlabelled antibody peroxidase-antiperoxidase method for the detection of IgG, IgM, complement (C3 and C1q), fibrinogen and albumin was applied to routinely processed paraffin sections of lung from 27 cases. The results in 11 cases were compared with those obtained by immunofluorescence using frozen sections. Tissue was obtained from surgical specimens of cases with interstitial pneumonia comprising 10 of the usual type (UIP) and three of the desquamative type (DIP). Tissue was also obtained from the specimens of cases with sarcoidosis (two cases) and granulomatous inflammation of unknown cause (one case).

There were 11 control cases, nine with primary carcinoma of the lung and two with metastatic tumours of the lung.

Immunoglobulins of various types and complement were seen in diseased lung tissue. Although most of these deposits were probably due to a non-immunological mechanism there was evidence of the possible implication of immune complexes in three cases of UIP and in the interstitial pneumonia present in the two cases of sarcoidosis.

The immunoperoxidase technique is a more sensitive method than immunofluorescence and has the additional advantage of the easy identification of the precise sites of the various deposits.

Immunological mechanisms are known to be associated with various lung diseases.[1] In some of these, immune deposits have been demonstrated by immunofluorescence in tissue sections and this is thought to be useful in diagnosing and explaining the pathogenesis of such diseases. Examples include Goodpasture's syndrome,[2-10] hypersensitivity pneumonitis,[8 11-13] idiopathic interstitial pneumonia,[8 14-16] fulminating interstitial pneumonia,[17] and lung diseases associated with rheumatoid arthritis,[18] systemic lupus erythematosus,[19-26] angioimmunoblastic lymphadenopathy,[27] chronic active hepatitis[28] and idiopathic thrombocytopenic purpura.[29]

Immunofluorescence entails the use of frozen sections and specially equipped microscopes. The fluorescence may fade; it is sometimes difficult to identify the exact site of the deposit, and the size of each block of tissue that can easily be examined is relatively small. Immunoperoxidase techniques are supposed to be devoid of all these drawbacks and are now widely used for the detection of various antigens in different tissues.[30-31] Recently, Inoue *et al*[26] have successfully used an immunoperoxidase method for the detection of immunoglobulins, complement and DNA in frozen sections of lung tissue from two patients with systemic lupus erythematosus. We report the results of an investigation into the feasibility of using an immunoperoxidase technique for the detection of immunoglobulins, complement and fibrinogen in fixed, routinely processed paraffin sections of lung. The results obtained by the immunoperoxidase technique were compared with those obtained by immunofluorescence.

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Material and methods

Details of the patients from whom the specimens were obtained are given in the Table. The tissues examined were all obtained at thoracotomy; either biopsy, lobectomy or pneumonectomy specimens (Table). The classification of interstitial pneumonia follows that described by Liebow and Carrington;[32] the desquamative type (DIP) and the usual type (UIP) which corresponds to the mural type as described by Scadding and Hinson.[33] Cases 17-27 acted as controls. The tissue examined from

Clinical, histological and immunohistological findings

Case no	*Age (yr) and sex*	*Nature of specimen*	*Histological* diagnosis*	*Associated disease*	*Activity*
Cases					
1	29M	Biopsy	UIP	Rheumatoid arthritis	+
2	65M	Biopsy	UIP	Rheumatoid arthritis	+
3	53M	Lobectomy	UIP, Rheumatoid nodule in pleura	Rheumatoid arthritis	+
4	76F	Biopsy	UIP, Liquid paraffin pneumonia	Rheumatoid arthritis	+
5	63M	Biopsy	UIP, Bronchiolitis obliterans		+
6	54M	Biopsy	UIP, Bronchiolitis obliterans		+
7	27M	Lobectomy	UIP	Bronchiectasis	+
8	65M	Lobectomy	UIP	Lung abscess	+
9	62F	Lobectomy	UIP, Malignant carcinoid of lung		+
10	47F	Biopsy	Honeycomb lung		−
11	59M	Biopsy	DIP		±
12	54M	Biopsy	DIP		+
13	33M	Biopsy	DIP		+
14	58M	Biopsy	Sarcoidosis		+
15	28M	Biopsy	Sarcoidosis		+
16	50M	Biopsy	Granulomatous inflammation of unknown cause		+
Controls					
17	43M	Lobectomy	Focal fibrosis	Carcinoma of bronchus, bronchiectasis	±
18	53F	Lobectomy	Fibroelastic scar	Carcinoma of bronchus	−
19	53M	Lobectomy	Pleural fibrosis	Carcinoma of bronchus, silicosis	−
20	66M	Lobectomy	Bronchiectasis, focal fibrosis	Carcinoma of bronchus	±
21	62F	Lobectomy	Normal	Carcinoma of bronchus	−
22	63M	Pneumonectomy	Normal	Carcinoma of bronchus	−
23	69M	Pneumonectomy	Normal	Carcinoma of bronchus	−
24	59M	Pneumonectomy	Normal	Carcinoma of bronchus	−
25	61M	Lobectomy	Normal	Scar carcinoma of lung	−
26	41F	Lobectomy	Normal	Metastatic choriocarcinoma	−
27	33M	Lobectomy	Normal	Metastatic testicular teratoma	−

*In sections used for immunological examination.

IgG						*IgA*						*IgM*						*C3*						*C1q*						*Fibrinogen*						*Albumin*					
1	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*	*1*	*2*	*3*	*4*	*5*	*IF*
Cases																																									
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	±	−	−	+	−	−	+	±	−	+	+	−	ND
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−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	ND
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Controls																																										
+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	+	+	+	+	+	−	ND
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Sites of immune deposits as seen by immunoperoxidase technique:
1 = linear alveolar deposits.
2 = deposits around alveolar macrophages.
3 = intravascular deposits.
4 = intra-alveolar and/or intrabronchiolar and/or intrabronchial deposits.
5 = positively stained plasma cells.
IF = results of immunofluorescent tests:
+ = when fluorescence was seen anywhere in the section.
− = when fluorescence absent.
ND = not done.

cases 21-27 was taken from normal parts of the lung some distance away from the carcinoma or metastatic deposits. The histological diagnosis (Table) was made on haematoxylin and eosin stained sections taken from the same blocks as that used for the immunoperoxidase tests.

The activity of interstitial pneumonia present was graded according to the intensity of the inflammatory reaction (macrophages, plasma cells, lymphocytes, neutrophils and eosinophils) in the interstitial tissue. This was done by two observers without knowledge of the immunological results. Grade + indicated the presence of many inflammatory cells, ± moderate numbers and − none, or only an occasional cell (Table).

IMMUNOPEROXIDASE TECHNIQUE (IP)

The tissues were fixed in 10% formalin for at least 24 h. Routine paraffin embedding was carried out and 5 μm sections cut and kept at 37°C in an incubator for a minimum of 24 h. Sections were then dewaxed and hydrated to Tris-buffered saline pH 7·6 (TBS). An unlabelled antibody (peroxidase-antiperoxidase) technique after trypsinisation was then carried out.

The sections were incubated at 37°C for 30 min in 0·1% solution of trypsin in 0·1% calcium chloride in TBS freshly prepared and preheated to 37°C just before use, and then washed with gentle agitation in TBS for 5 min. The endogenous peroxidase was blocked by applying freshly prepared 0·5% hydrogen peroxide in methanol for 30 min at room temperature, followed by washing with agitation in three ten minute changes of TBS. Non-immune normal swine serum, diluted 1/10 TBS, was applied for 10 min and then drained. Specific rabbit antihuman serum was applied for 30 min in a moist chamber at room temperature.

In the first five cases, various concentrations of antisera were tried and the highest concentration that gave a positive result without background staining was subsequently used. These were 1/1000 for anti-IgG, IgA and IgM, 1/500 for C3 and fibrinogen, 1/100 for C1q and 1/8000 for albumin. The sections were then washed in TBS for 10 min with agitation and this was repeated three times. Swine antirabbit immunoglobulin, diluted 1/100 with TBS, was applied for 30 min in a moist chamber at room temperature. This was followed by three washes of 10 min each with TBS and then peroxidase-antiperoxidase complex (PAP) diluted 1/100 with TBS was applied for 30 min in a moist chamber at room temperature. After three ten minute washes with TBS, DAB reagent (see below) was applied for 10 min.

Sections were then washed in running tap water for 3 min. The sections were counterstained with haematoxylin for 2 min, washed in tap water for one minute, differentiated in 1% acid alcohol, 2-3 dips, and "blued" in running tap water for 10 min. After dehydration, the sections were cleared and mounted in a synthetic resin. The peroxidase reaction site was dark brown and nuclei blue. Positive and negative controls were included with each tested set of slides. Positive controls were sections of tonsillar tissue known to be positive for all classes of immunoglobulins being used. Negative controls were sections of test lung tissue in which the step using specific antihuman antiserum was omitted.

REAGENTS

(a) DAB: 50 mg 3,4,3′,4′ tetra-aminobiphenyl-hydrochloride dissolved in 100 ml TBS, then filtered; 0·1 ml 30% (100 vols) hydrogen peroxide was added just before use.

(b) Trypsin 1/250 obtained from Difco Laboratories, Surrey, England.

(c) All specific sera and antisera used were Dako products obtained from Mercia Brocades Ltd, Surrey, England.

IMMUNOFLUORESCENT TECHNIQUE (IF)

A piece of unfixed lung tissue approximately 1·0 × 0·5 × 0·5 cm taken immediately after surgical removal was mounted with Tissue-Tek 11 OCT compound (Miles Laboratories Incorporated, USA) on to cork mats and snap-frozen with CO_2 gas. Serial sections of the frozen tissue block were cut on a cryostat at a thickness of about 6 μm. Two serial sections were mounted on each of 10 clean slides and allowed to dry at room temperature for 30 min and then fixed in acetone for 15 min and dried in air. The sections were washed thoroughly in phosphate-buffered saline pH 7·6 (PBS) for 10 min and specific rabbit antihuman sera conjugated with fluorescein isothiocyanate (FITC) diluted 1/10 with PBS was added and sections kept in a moist chamber for 30 min. They were then rinsed with PBS and thoroughly washed with two changes of PBS of one hour each. The sections were mounted in a 10% solution of glycerol in PBS. The slides were examined with a Leitz Dialux microscope using transmitted ultraviolet light, dark ground condenser, exciter filters BG38 and KP490 and barrier filter interference green S525. The reaction sites showed bright green fluorescence. The controls were similar to those used for the immunoperoxidase technique.

All the sera used were obtained from Hoechst UK Limited.

Results

The summary of the findings are given in the Table.

With the immunoperoxidase technique for immunoglobulins, complement, fibrinogen and albumin, brown deposits were found lining alveoli and within alveolar walls (Fig. 1), around intra-alveolar macrophages (Fig. 2), within lumina of bronchi, bronchioli, and alveoli (Fig. 3), and within blood vessels (Fig. 4). Brown deposits were only found in plasma cells with the antisera of immunoglobulins (Fig. 5).

Sections of tonsillar tissue used as positive controls with immunoperoxidase always contained cells positive for the three classes of immunoglobulin but not for complement or fibrinogen. Lung sections in which the specific antisera were omitted did not stain and brown deposits in plasma cells present in most lung sections examined were only found with the antisera of immunoglobulins.

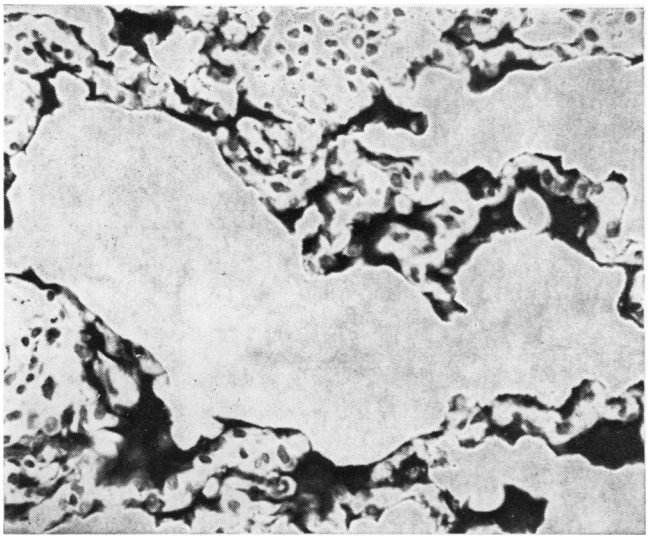

Fig. 1 *Case 1. Linear deposits of IgG lining and within alveolar walls. Immunoperoxidase* × *300*

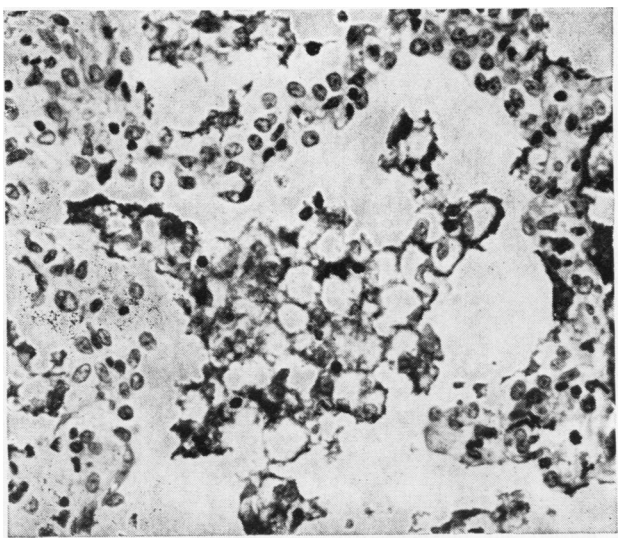

Fig. 2 *Case 4. Deposits of IgA around intra-alveolar macrophages. Immunoperoxidase* × *300.*

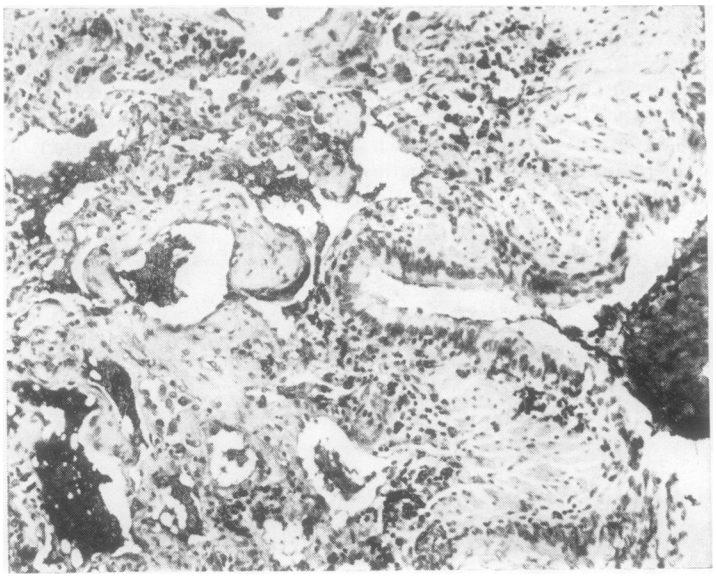

Fig. 3 *Case 9. Intra-alveolar and intrabronchial deposits of IgG. Immunoperoxidase × 120.*

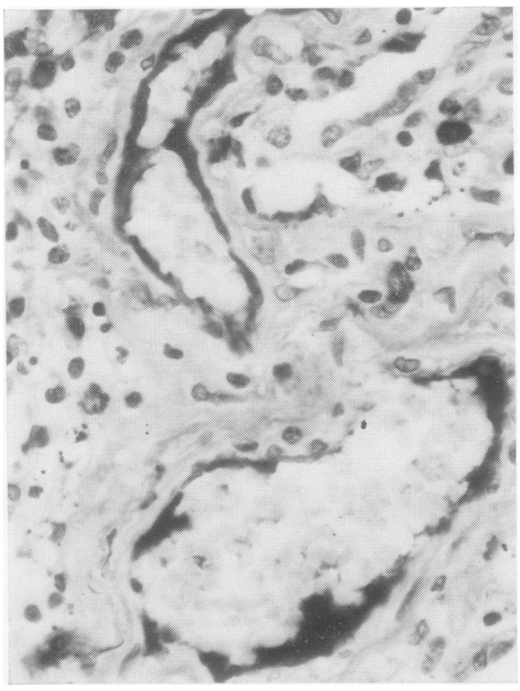

Fig. 4 *Case 9. Intravascular deposit of IgG. Immunoperoxidase × 500.*

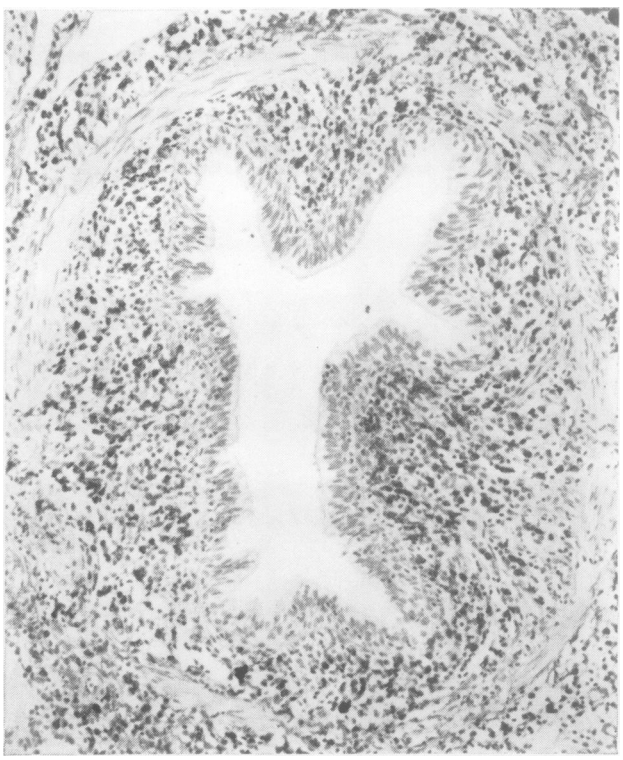

Fig. 5 *Case 1. Deposits of IgM in peribronchial plasma cells. Immunoperoxidase × 120.*

Discussion

Although the contrast between specific staining and background was sharper with IF the exact site of the deposits was more accurately identified with IP because the deposits were seen against a clearly identifiable background of tissues and cells. This is an important point in a tissue such as the lung which has a complex histological structure and thus many sites at which immune deposits may occur. Until now the significance of immune deposits, as demonstrated by IF, has been marred by the difficulty of their interpretation and exact localisation[8] as well as by the necessity of using frozen sections of freshly removed tissue which limits the number of cases that can be examined. IP provides a method for the detailed study of a large number of cases, both retrospectively and prospectively.

The specificity of IP was confirmed by the controls used and by comparing the results obtained in 11 cases by this method with those obtained by IF (Table). All immune deposits seen with IF were positively stained by IP, but occasionally immune deposits, complement, and fibrinogen were found with IP and not by IF. These deposits seen by IP are unlikely to be the result of a non-specific or cross reaction because of the controls used and because of the complete absence of background staining.

Our results clearly show that in normal lung tissue (cases 21-27) immunoglobulins, complement, fibrinogen and albumin may sometimes be seen within blood vessels. The presence of immune deposits in other sites within the lung is always associated with disease. There are two possible mechanisms to account for extravascular immunoglobulins in diseased lung tissue. The first is non-specific and is due to exudation of serum proteins from blood vessels in inflamed tissues. The presence of albumin, a protein not involved in any immunological reaction, at the same sites as the immunoglobulins is evidence that this is the explanation in many of our cases (Table). The other mechanism is that an immunological reaction has occurred which is suggested by the presence of complement. However in cases in which C3 is present without C1q it is possible that complement could have been activated via the alternate pathway by aggregated IgA which may be present in the lung. Only in those cases in which C1q is present, usually with C3, can we suggest that an immunological mechanism may have occurred as C1q can only be formed by the activation of complement by immune complexes via the classic pathway. There were only three cases of UIP (cases 1, 6, 9) and two of sarcoidosis (cases 14, 15) in which C1q, C3, and IgG or IgM were apparently present at the same sites. Thus these are the only cases in which we think immune complexes may have been present. This is in agreement with other authors who have suggested that UIP may be an immune complex disease.[1 15 19]

Although we have only examined two cases of sarcoidosis our findings of deposits of possible immune complexes in these cases are intriguing. Immune complexes have been found in the circulation in patients with active sarcoidosis[34-36] and in one case of sarcoidosis by IF within and around granulomata in the lung.[37] In our cases using IP the deposits were confined to the lung parenchyma in areas of interstitial pneumonia and were not within the granulomata. Thus it is possible that the interstitial pneumonia often seen in sarcoidosis may also be an immune complex-mediated process.

Our studies have shown that IP is a reliable method for the identification of immunoglobulins, complement and fibrinogen in the lung. This technique should make it easier to clarify the diagnostic and possible pathogenic significance of immune deposits in lung disease.

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