EXTENDED REPORTS

Detection of immune deposits in skin lesions of patients with Wegener's granulomatosis

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Abstract

***Background*—Wegener's granulomatosis (WG) is considered a pauci-immune systemic vasculitis based on the absence of immune deposits in renal biopsies of patients with active disease. In animal models of antineutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis, immune deposits along the glomerular capillary wall are present at early stages of lesion development. These deposits are degraded rapidly, resulting in "pauci-immune" lesions.**

***Objective*—To test the hypothesis that immune deposits can also be detected in early lesions of patients with WG, thereby initiating an inflammatory reaction that, in time, is augmented in the presence of ANCA, resulting in pauci-immune lesions later on.**

***Methods*—The presence of immune deposits in skin biopsies taken within 48 hours of lesion development was investigated. Direct immunofluorescence was used to examine 32 skin biopsies for the presence of immune deposits (IgG, IgA, IgM, C3c). When possible, a comparison was made between the immunofluorescence findings in renal and skin biopsies taken at the same time.**

***Results*—Four of 11 biopsies taken at initial presentation and four of 21 biopsies taken at the onset of a relapse of WG showed IgG and/or IgA containing immune deposits in the subepidermal blood vessels. All nine renal biopsies showed pauci-immune glomerulonephritis, irrespective of the presence (n=5) or absence (n=4) of immune deposits in the skin biopsy.**

***Conclusion*—A substantial number of skin biopsies showed immune deposits during active disease. These results could support the hypothesis that immune complexes may trigger vasculitic lesions in WG.**

(*Ann Rheum Dis* 2001;**60**:1097–1102)

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Accepted 14 May 2001

Wegener's granulomatosis (WG) is a form of systemic vasculitis characterised by granulomatous inflammation involving the respiratory tract, necrotising vasculitis affecting small to medium sized blood vessels, and necrotising crescentic glomerulonephritis.[1]

The pathogenesis of WG is still unknown. There are several indications that antineutrophil cytoplasmic antibodies (ANCA) have a pathophysiological role in WG.[2] Firstly, a rise in ANCA level precedes clinical disease activity in many patients.[3–5] Secondly, in vitro experiments show that ANCA can activate primed neutrophils to release reactive oxygen species and lytic enzymes.[6 7] Thirdly, antibodies directed against myeloperoxidase (MPO-ANCA) aggravate mild antiglomerular basement membrane (GBM) mediated glomerular injury in the rat.[8] Furthermore, in vivo experimental models show that in rats immunised with MPO, a renal perfusion or systemic injection of a neutrophil extract, in combination with H_2O_2, results in necrotising glomerulonephritis and vasculitis, respectively.[9 10] Immune deposits are found in the vessel wall at a very early stage of lesion development in these animal models, but disappear at a later stage of the disease. Evidence for the presence of immune deposits in patients with WG is, however, scarce. In 1982, Shasby *et al* described granular deposition of IgG and complement in pulmonary lesions of a patient with WG.[11] In contrast, in most renal biopsies from patients with necrotising crescentic glomerulonephritis due to WG, immune deposits are rarely found. Hence, these renal lesions are commonly described as being "pauci-immune".[12–15]

In this study we examine skin biopsies from patients with WG taken at initial disease manifestation and at the onset of relapses of WG for the presence of immune deposits. These biopsies were taken from newly developing skin lesions. When available, we compared immunofluorescence (IF) findings from the skin biopsy with IF findings of the renal biopsy taken simultaneously from the same patient.

Patients and methods

We examined our database from 1983 to 1998 for patients with proteinase 3 (PR3)-ANCA associated WG who fulfilled both the classification criteria of the American College of Rheumatology[16] and the Chapel Hill Consensus Conference definition.[17] Patients included in this study had to have undergone a skin biopsy during an active phase of the disease that was tested for the presence of immune deposits using direct IF. Twenty three patients, all white subjects, met these criteria. Biopsies of these 23

Table 1 Clinical, histopathological and immunofluorescence (IF) findings of skin and renal biopsies in newly diagnosed patients with Wegener's granulomatosis

Patient No	Sex	Age at time of biopsy	Clinical diagnosis of skin lesion	Histopathology of skin lesion	Immune deposits in blood vessel wall of skin biopsy specimen	Rheumatoid factor	Organ involvement	IF findings of renal biopsy
1	M	27	Purpura	LCV	IgG + IgM + IgA + C3c + fibrin	Neg	ENT, L, K, S	NB
2	M	27	Purpura	LCV	Fibrin	Neg	ENT, S	NB
3	M	65	Purpura	LCV	C3c + fibrin	Neg	L, K, S	NB
4	M	72	Purpura	NS	—	Neg	ENT, L, K, S	NB
5	M	33	Purpura	NS	IgG + IgM + IgA + C3c + fibrin	Neg	ENT, L, K, E, S	Pauci-immune
6	F	59	Purpura	NS	—	Neg	ENT, L, K, J, S	No IF data
7	F	79	Purpura	LCV	—	Pos	ENT, K, J, S	NB
8	F	23	Purpura	NS	IgG + IgM + C3c + fibrin	Neg	ENT, L, K, S	Pauci-immune
9	M	58	Purpura	LCV	IgG + C3c + fibrin	Pos	ENT, L, K, J, S	Pauci-immune
10	M	66	Purpura	LCV	C3c + fibrin	Neg	ENT, K, PNS, S	NB
11	F	28	Nodule	GRAN	—	Neg	Unknown	Pauci-immune

M = male; F = female; LCV = leucocytoclastic vasculitis; GRAN = granuloma annulare; NS = non-specific inflammation; ENT = ear, nose, and throat; L = lung; K = kidney; J = joints; PNS = peripheral nervous system; E = eyes; S = skin; NB = not biopsied at the same time.

patients (32 biopsies) were selected for this study. They were taken either within two weeks of initial presentation of WG (11 patients; 11 biopsies) or within two weeks of an onset of relapses of WG (13 patients; 21 biopsies). All biopsies were taken from active lesions, usually on arms (n=12), legs (n=13) or feet (n=4). In some patients, biopsies were taken from other places, such as groin (n=2) or scalp (n=1). Tables 1 and 2 present other patient characteristics. These biopsies were not selected and were obtained from lesions with different degrees of severity. In 16 cases a concomitant biopsy of clinically normal skin was taken.

Biopsies were taken and stained according to a previously described protocol[18 19] with minor modifications. In short, after local anaesthesia with lidocain (lignocaine), a 3–4 mm punch biopsy was obtained from a skin lesion within 48 hours of development of the lesion. All biopsies were snap frozen in liquid nitrogen and kept in cold storage (−80°C) until further processing, which occurred within a week. Four micrometre tissue sections were cut in a cryostat at –23°C, collected on 2% Silane coated glass slides (3-aminopropyl triethoxysilane; Sigma Aldrich Co Ltd, Irvine, UK), air dried for 30 minutes in front of a fan, and used unfixed in IF staining procedures. Sections were incubated with fluorescein isothiocyanate (FITC) conjugated, Fc-specific $F(ab)_2$ antisera (Protos Immunoresearch, Burlingame, CA, USA) directed against IgG (1:100 diluted), IgM (1:40 diluted), and IgA (1:60 diluted). The presence of complement C3c and fibrin(o-gen) was detected using rabbit anti-C3c antisera (1:100 diluted, Dakopatts, Copenhagen, Denmark) and rabbit antifibrin(ogen) antisera (1:80 diluted, Dako), respectively. All antisera were diluted in phosphate buffered saline (PBS, pH 7.3) supplemented with 1% bovine serum albumin (Sigma). Sections were rinsed in PBS and a nuclear counterstaining using bisbenzimide (Boehringer Mannheim, Heidelberg, Germany) was performed. Phosphate buffered glycerol (1:1 vol/vol, pH 7.3) was used to mount sections, which were subsequently examined with a fluorescence microscope (Leitz Orthoplan) equipped with a Xenon arc (XBO, 75 W) and epi-illuminator for incident light excitation.

Light microscopy of these skin biopsies was performed on haematoxylin-eosin stained, paraffin embedded sections.

When a renal biopsy was available within a 2 week time period from the time of the skin biopsy, the IF findings of the renal and skin biopsies were compared.

IF of the renal biopsy was performed on unfixed, snap frozen renal tissue, stained with FITC-labelled anti-IgG, anti-IgM, anti-IgA or anti-C3 antibodies (all diluted 1:100, Dako). Scoring was performed as previously described.[20]

The presence of PR3-ANCA was confirmed both by the indirect IF technique[21] and by an antigen-specific enzyme linked immunosorbent assay (ELISA).[20]

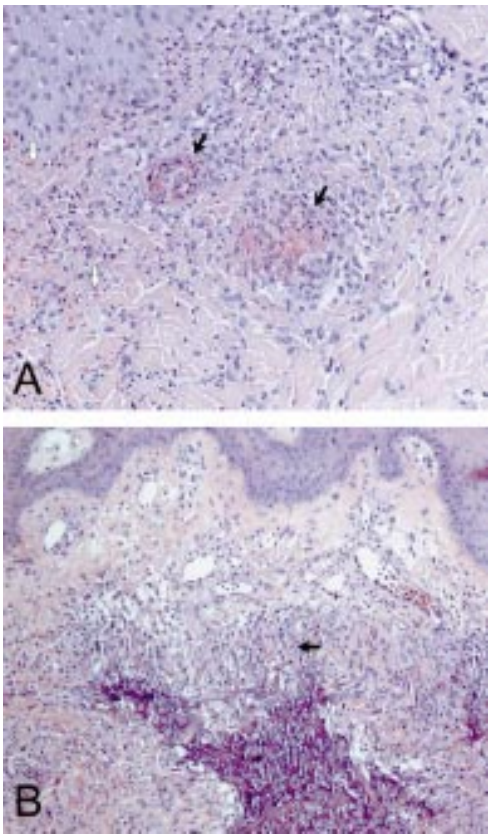

Figure 1 (A) Leucocytoclastic vasculitis. There is infiltration of vessel walls with neutrophils and fibrinoid necrosis (black arrows) with leucocytoclasia and extravasation of red blood cells (white arrows) (haematoxylin and eosin, objective lens ×50). (B) Cutaneous granuloma annulare with large area of "necrobiosis" surrounded by a palisade of histiocytes (black arrow) (haematoxylin and eosin, objective lens ×25).

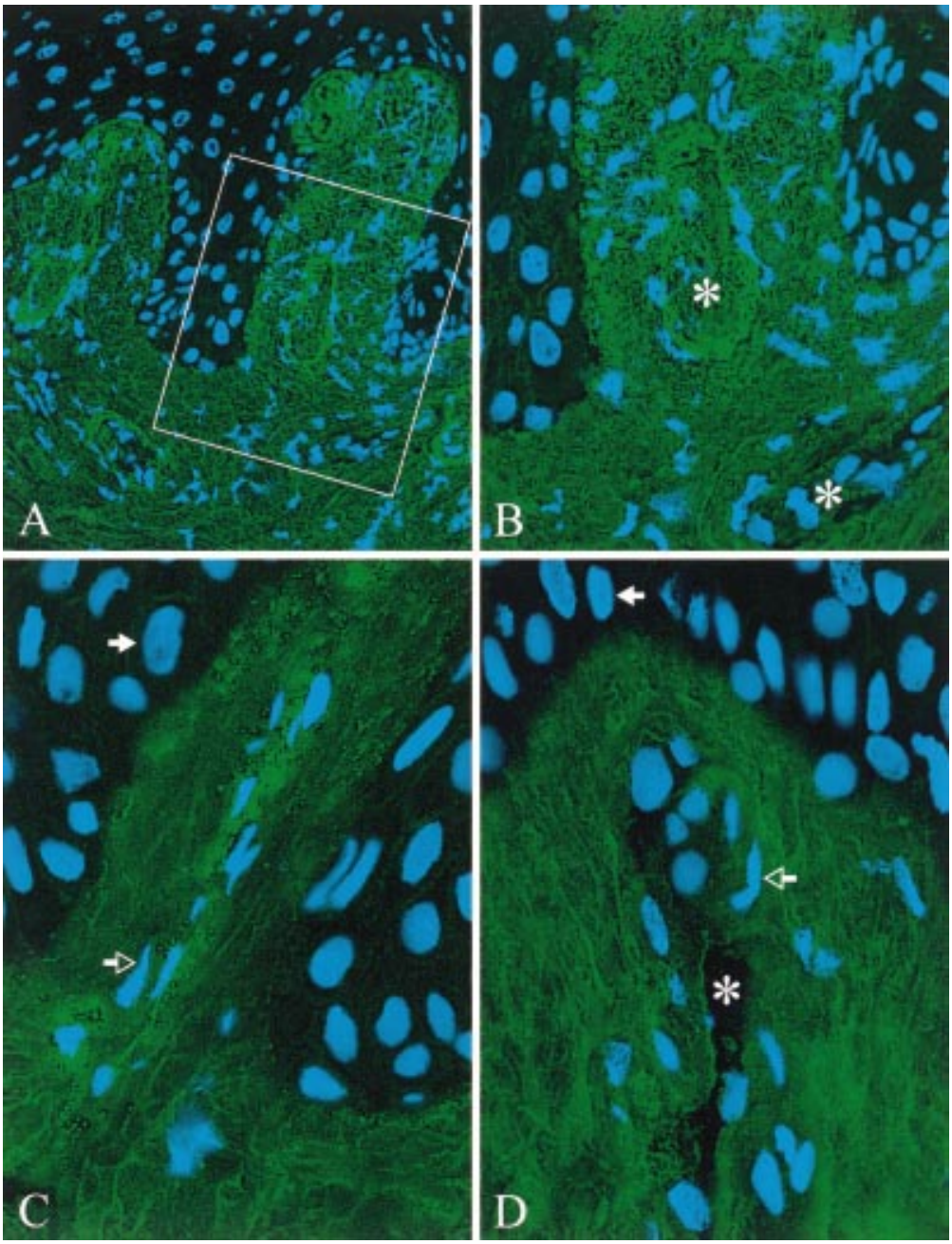

Figure 2 Immunofluorescence images of frozen skin sections from patients with Wegener's granulomatosis, counterstained with blue fluorescent bisbenzimide to visualise cell nuclei. Asterisks indicate the lumen of small blood vessels, the closed arrows point towards epidermal cell nuclei, and open arrows point towards endothelial cell nuclei. (A) Overview of the epidermis and superficial dermis in patient No 5 showing extensive granular staining for IgG in and around blood capillary walls in dermal papillae (objective lens ×40). (B) Higher magnification of the inset on figure A (objective lens ×20). (C) Blood capillary wall of dermal papilla of patient No 13 showing fine granular staining for IgA (objective lens ×40). (D) Blood capillary in dermal papilla of perilesional skin from patient No 4 showing negative staining for IgA (objective lens ×40).

Results

INITIAL PRESENTATION

Table 1 presents clinical, histopathological, and IF findings of skin biopsies from 11 patients at the initial presentation of WG. In all but one patient the clinical presentation of the skin lesion was purpura; the remaining patient had a nodule on the elbow. Light microscopic examination showed leucocytoclastic vasculitis in 6/10 patients with purpura, whereas granulomatous inflammation without vasculitis was present in the patient with the nodule (fig 1). The other biopsies showed non-specific inflammation.

Four of 11 patients showed immunoglobulin deposits in the subepidermal blood vessel walls in the diseased skin. IgG, IgM, and IgA deposits were detected in four, three, and two patients, respectively. The IgA IF pattern seen in the biopsies of the two patients with WG was indistinguishable from IgA staining seen in patients with Henoch-Schönlein purpura (fig 2). Immune deposits were not found in biopsies taken from clinically normal skin of newly diagnosed patients with WG (n=5).

Complement C3c deposits were detected in the blood vessel walls of six patients, whereas fibrin deposits were detected in seven patients.

Table 2 Clinical, histopathological, and immunofluorescence (IF) findings of skin and renal biopsies in patients at the onset of relapse of Wegener's granulomatosis

Patient No	Sex	Age at time of biopsy	Clinical diagnosis of skin lesion	Histopathology of skin lesion	Immune deposits in blood vessel wall of skin biopsy specimen	Rheumaoid factor	Organ involvement	IF findings of renal biopsy
2	M	27	Purpura	Unknown	C3c + fibrin	Neg	ENT, S	NB
12	M	26	Purpura	LCV	—	Pos	ENT, K, PNS, S	NB
		27	Nodule	NS	—	Pos		NB
		30	Nodule	NS	Fibrin	Neg		NB
13	M	69	Purpura	LCV	IgA + fibrin	Pos	ENT, L, K, J, PNS, S	NB
14	M	57	Purpura	LCV	—	Neg	ENT, L, K, E, S	NB
15	M	73	Ulcer	NS	IgG + C3c + fibrin	Neg	ENT, K, J, S	No IF data
16	F	70	Purpura	NS	—	Neg	ENT, K, J, S	Pauci-immune
17	F	21	Nodule	NS	IgM + C3c	Neg	ENT, L, K, J, S	NB
18	M	35	Purpura	LCV	IgG + IgM + C3c + fibrin	Pos	ENT, L, J, PNS, S	Pauci-immune
		38	Purpura	LCV	IgG + IgM + C3c + fibrin	Neg	ENT, L, K, J, E, S	NB
19	M	63	Nodule	GRAN	—	Neg		NB
20	M	76	Purpura	LCV	IgM + C3c	Pos	ENT, K, J, PNS, S	Pauci-immune
21	M	69	Purpura	NS	Fibrin	Neg	L, K, E, S	Pauci-immune
22	M	48	Purpura	GRAN	—	Neg	ENT, L, K, J, S	NB
		50	Nodule	GRAN	—	Neg		NB
		52	Nodule	NS	IgM + C3c + fibrin	Neg		NB
		53	Purpura	GRAN	—	Neg		NB
		56	Nodule	NS	—	Neg		Pauci-immune
23	F	38	Purpura	LCV	C3c + fibrin	Neg	K, J, PNS, S	NB
		39	Purpura	LCV	—	Pos		NB

M = male; F = female; LCV = leucocytoclastic vasculitis; GRAN = granuloma annulare; NS = non-specific inflammation; ENT = ear, nose, and throat; L = lung; K = kidney; J = joints; PNS = peripheral nervous system; E = eyes; S = skin; NB = not biopsied at the same time.

C3c and fibrin deposits were not only detected in blood vessel walls of biopsies obtained from diseased skin but also in 1/5 biopsies obtained from clinically normal skin of newly diagnosed patients. The presence of C3 and fibrin in the absence of immune deposits indicates vessel reactivity and was seen in two patients. Immunoglobulin deposits were detected both in skin lesions histopathologically showing leucocytoclastic vasculitis (2/6) and in lesions showing non-specific inflammation only (2/4). The biopsy showing granulomatous inflammation without vasculitis showed no immune deposits.

In 4/11 patients, renal biopsies were carried out at the same time as the skin biopsies. All four biopsies showed necrotising crescentic glomerulonephritis. In three patients the renal biopsy showed a pauci immune pattern contrasting with immune deposits found in the skin biopsy, whereas in one patient a pauci-immune pattern was found in both the kidney and the skin biopsy (table 1).

RELAPSE

Table 2 presents the findings in 21 skin biopsies taken from 13 patients at a relapse of WG. Clinical presentation of the skin lesion was purpura, nodule, or ulcer. Histopathology of the purpura lesions (n=13) showed leucocytoclastic vasculitis in eight biopsies, granulomatous inflammation in two biopsies, two non-specific inflammation biopsies, and was not available in one case. Histopathology of the nodular lesions (n=7) showed granulomatous inflammation in two biopsies (fig 1), whereas non-specific inflammation was found in the remaining five biopsies. The histopathology of the ulcerous lesion showed non-specific inflammation.

Immunoglobulin deposits in subepidermal blood vessel walls were detected in 7/21 biopsies (6/13 patients). IgG and IgA were detected in three and one biopsy, respectively; IgM was detected in five biopsies. Complement C3c and fibrin deposits were detected in eight biopsies (seven patients) and nine biopsies (eight patients), respectively. Noting that IgM and C3 can be trapped non-specifically in injured blood vessels, we found definite proof of immune deposits in four biopsies in which either IgG or IgA was demonstrated.

In all 11 biopsies taken simultaneously from clinically normal skin at the time of relapse, IgA was found in one, whereas IgG deposits were not found. In addition, IgM, C3c, and fibrin deposits were found in three, four, and three biopsies, respectively.

Immune deposits were not only detected in blood vessel walls but occasionally also in other areas of the skin, such as the epidermal basement membrane zone or the dermis (fig 2).

All biopsies showing granulomatous inflammation, two biopsies from purpuric lesions, and two from nodular lesions, did not reveal any immune deposits.

In five patients renal biopsies were carried out at the same time as the skin biopsy. In all five biopsies necrotising crescentic glomerulonephritis was found with a pauci-immune pattern. In two of those five cases immune deposits were found in the skin biopsy, while a pauci-immune pattern was found in both the kidney and the skin biopsy of three patients (table 2).

Discussion

This study shows the presence of IgG- and/or IgA-containing immune deposits in blood vessel walls of skin biopsies in 4/11 biopsies of patients with newly diagnosed WG and in 4/21 biopsies of patients with relapses of WG. Renal biopsies taken at the same time did not show immune deposits. Immune deposits in the skin were found mainly in subepidermal blood vessel walls, but occasionally also along the epidermal basement membrane zone or in the dermis.

IgA in immune deposits in patients with WG has previously been described as present in renal biopsies.[22 23] Andrassy *et al* showed that patients with WG in remission could sometimes develop de novo IgA nephropathy, not to be mistaken with a relapse of WG.[24]

However, in most studies no immune deposits were found in biopsies from patients with WG.[12–14 20 25] We considered the possibility that these biopsies, which were obtained from the kidneys in most cases, were taken from lesions in which invading leucocytes had degraded the immune deposits. Similar mechanisms have been described in an Arthus-reaction animal model of vasculitis.[26 27] In this animal model, neutrophils degrade the immune deposits within 18–48 hours after deposition.

We therefore postulated that the presence of immune deposits depends on the stage of development of the lesion and that in newly developing skin lesions, leucocytes may not yet have degraded the immune deposits, because these biopsies are taken at the time lesions develop.

In only a few case reports and small sized studies has the presence or absence of immune deposits in skin biopsies of patients with WG been described.[25 28–30] In none of these studies, however, were IF findings in skin biopsies of patients with WG the primary goal of the investigation. These studies demonstrated immune deposits in 16/23 (70%) reported cases. Importantly, four of these 23 patients had IgG and IgA deposits. In only one study[29] were IF data in the skin of two patients compared with IF data found in concomitantly obtained renal biopsies. Immune deposits in both skin and renal biopsy were detected in one patient, whereas the other patient showed no immune deposits in the skin but IgG and IgA deposits in the renal biopsy.

These earlier studies and our current results may shed new light on the question whether WG is a genuine pauci-immune vasculitis. This concept is based mainly on studies describing renal biopsies of patients with WG in which immune deposits are rarely seen.[12–15] Indeed, in the present study we show that immune deposits may be present in skin lesions, while absent in renal lesions. However, because electron microscopy was not performed on these renal biopsies we cannot conclude unequivocally that immune complexes were absent at the time of biopsy as reabsorbed immune complexes might have been observed by electron microscopy. Likewise, we cannot exclude unequivocally that "non-specific trapping" of immunoglobulins in skin vessels occurs as a result of vessel wall damage. However, in our view it is unlikely that this is the case because deposits of immunoglobulins of the class that probably cause trapping—that is, IgM, are only detected in a minority of our biopsies, whereas damaged vessels were present in most of them. In our study we found immune deposits in skin but not in renal biopsies. Immune deposits are also occasionally detected in pulmonary biopsies[11] and renal biopsies of patients with WG.[6 12 31 32] Thus we conclude that in contrast with current thinking, immune deposits can be found in, at least, a subset of patients with WG.

Because immune deposits were present in a substantial number of our patients with active WG we suggest that WG may start as an immune complex mediated vasculitis in this subset of patients with WG. The paucity of immune complexes, as found in renal biopsies, may be the result of degradation of immune complexes after deposition in the glomeruli.

In patients with WG we previously proposed that the presence of ANCA might aggravate an initial immune response, resulting in an accelerated degradation of immune deposits.[33] An animal model for WG also corroborates this concept,[9] because in rats immunised with MPO, IgG and C3 immune deposits could be detected along the GBM 24 hours after injection of a lysosomal extract and H_2O_2, whereas after four days, when renal lesions were maximal, the immune deposits were no longer present.

There are several mechanisms by which immune deposits can be formed in the blood vessels of patients with WG. Circulating immune complexes can be deposited in small blood vessels such as arterioles and venules. There have been speculations about circulating immune complexes in patients with WG,[14 34–36] but the presence of these circulating immune complexes is still debated. Another possibility is the in situ formation of immune complexes due to the deposition of cationic proteins on negatively charged surfaces such as the GBM of the kidneys. Cationic proteins relevant in the pathophysiology of WG can be human proteins such as PR3 and MPO,[6] both of which are ANCA antigens. Other possible candidates may be cationic proteins from bacteria relevant to the pathophysiology of WG, such as *Staphylococcus aureus*.[33 37] Animal models have shown that at least two staphylococcal cationic proteins can be deposited at the GBM and cause glomerulonephritis.[38–40] In vitro studies have confirmed that one of these cationic proteins, staphylococcal acid phosphatase, binds to endothelial cells through charge interaction.[33] Staphylococcal acid phosphatase could, consequently, act as a planted antigen, resulting in in situ formation of immune complexes.

Finally, our study shows that in a clinical setting a skin biopsy is not helpful in differentiating WG from other conditions in which both glomerulonephritis and skin vasculitis occur, such as systemic lupus erythematosus, Henoch-Schönlein purpura, cryoglobulinaemia and endocarditis, because immune deposits may be present in all these conditions. Importantly, the presence of IgA and/or other immunoglobulins in skin biopsies does not exclude a diagnosis of WG. This latter finding challenges the current practice in patients with purpura, arthralgias, and glomerulonephritis of making a diagnosis of Henoch-Schönlein purpura based on the demonstration of IgA deposits in skin biopsies only.[41]

The results presented here show that immune deposits can be detected in skin biopsies taken at initial presentation and at the onset of relapses in a subset of patients with WG while, at the same time, renal biopsies are pauci-immune. Further studies are in progress to determine which antigens play a part in these immune deposits.

JD Elema and ATMG Tiebosch are acknowledged for reviewing renal biopsies, HJ Meijer and J Zuiderveen for their technical

assistance, and PA Holloway for carefully reading this manuscript.

Part of this study was presented at the 9th international ANCA Workshop in Gröningen, The Netherlands and published as an abstract in Clin Exp Immunol 2000;120(suppl 1):47.

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