

Glomerular Monocyte-Macrophage Features in ANCA-Positive Renal Vasculitis and Cryoglobulinemic Nephritis

MARIA PIA RASTALDI,* FRANCO FERRARIO,* ANDOR CRIPPA,*
GIACOMO DELL'ANTONIO,[†] DONATELLA CASARTELLI,[‡] CARLO GRILLO,[‡] and
GIUSEPPE D'AMICO,*[§]

*Renal Immunopathology Center and [§]Division of Nephrology, San Carlo Borromeo Hospital, and

[†]Department of Pathology, San Raffaele Hospital, Milan; and [‡]Department of Nephrology, San Anna Hospital, Como, Italy.

Abstract. Although it is widely known that many macrophages are present in glomeruli of antineutrophil cytoplasmic antibody (ANCA)-positive renal vasculitis (ANCA+RV) and are believed to contribute to necrotizing extracapillary damage, their precise role is not yet completely understood, especially in humans. The goal of this study was to provide evidence of glomerular macrophage properties in human vasculitis. Twenty-five renal biopsies of ANCA+RV and 18 cases of cryoglobulinemic glomerulonephritis (cryoGN), a disease characterized by massive glomerular macrophage infiltration but absence of necrotizing extracapillary lesions, were selected, and macrophage number, adhesion, acute activation, proliferation, and apoptosis were analyzed by immunohistochemistry and *in situ* hybridization. Accumulation of macrophages in ANCA+RV was found in areas of glomerular active lesions, whereas in cryoGN, they homogeneously occupied the entire glomerular tuft. Considering the areas of accumulation, com-

parable macrophage numbers were detected in both diseases. Glomerular vascular cell adhesion molecule-1 was found only in ANCA+RV and only in areas of active lesions. Acute macrophage activation (HLA class II, 27E10) and proinflammatory cytokine production (tumor necrosis factor- α , interleukin-1 α) were prominent in ANCA+RV, whereas in cryoGN, 30% of glomerular macrophages seemed activated and cytokine expression was limited to a few glomerular cells ($P = 0.01$). Moreover, only in ANCA+RV proliferative markers were shown on glomerular macrophages and apoptotic macrophages were found. From the data, it seems that ANCA+RV and cryoGN differ profoundly in macrophage properties, namely adhesion, proliferation, and apoptotic clearance. Moreover, acute activation and cytokine production seem to be present in a greater number of macrophages in ANCA+RV, giving this disease a stronger severity that could be taken into account for therapeutic strategies.

Antineutrophil cytoplasmic antibody (ANCA)-associated renal vasculitis, namely Wegener's granulomatosis and microscopic polyarteritis and its renal limited variant, are characterized at renal biopsy by two major glomerular lesions with a great variability in their extent: necrosis of the tuft and extracapillary proliferation. They range from focal and segmental necrotizing extracapillary glomerulonephritis to the presence of massive necrosis and large circumferential crescents in more than 50% of glomeruli (1,2). Around damaged glomeruli, a characteristic periglomerular infiltration frequently is present, sometimes extremely intense and associated with rupture of Bowman's capsule, giving the lesion a granulomatous appearance and making it impossible to discriminate between intra- and extraglomerular infiltration (periglomerular granuloma) (1,2).

It is widely known, since it was demonstrated by Atkins *et*

al. in 1972 (3), that a high number of macrophages accumulate in necrotizing crescentic lesions, where they are considered to have a key role in amplifying glomerular inflammation and participating in crescent formation (4), although their precise properties are not yet completely understood, especially in human disease. Macrophages are indeed very heterogeneous, and specific actions are directly influenced by the particular microenvironment in which they are present (5,6).

Most glomerular macrophages are believed to be derived from blood monocytes, and their entrance is permitted by the glomerular endothelial expression of adhesion molecules, especially Ig-like molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), that establish a firm adhesion to the endothelium (7,8).

In glomeruli, monocytes become macrophages and when properly activated are able to produce an arsenal of chemical mediators with many different properties. Acutely activated macrophages can be recognized by overexpression of HLA class II antigens, *de novo* expression of 27E10 antigen, and production of proinflammatory products, such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) (9–11). Instead, mature macrophages do not possess the 27E10 epitope on their surface (11). Once a macrophage completes its action, its exit

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Correspondence to Dr. Maria Pia Rastaldi, Renal Immunopathology Center, Division of Nephrology, San Carlo Borromeo Hospital, Via Pio II, 3, 20153 Milan, Italy. Phone: 39240222343; Fax: 39240222222; E-mail: mp.rastaldi@oscb.sined.net

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is thought to depend mostly on lymphatic drainage, although apoptosis could be another possible mechanism of clearance (12).

To provide evidence of macrophage properties in ANCA-positive renal vasculitis (ANCA+RV) at time of renal biopsy, we conducted an immunohistochemical and *in situ* hybridization study, seeking various markers for the main steps of macrophage infiltration: adhesion to endothelium, acute activation, production of proinflammatory cytokines, proliferation, and apoptosis. Results were compared with those obtained in cryoglobulinemic glomerulonephritis (cryoGN), which occurs almost exclusively in type II mixed cryoglobulinemia (13). The disease has a typical morphologic picture of membranoproliferative exudative glomerulonephritis, with massive glomerular macrophage infiltration in most cases and macrophages mainly interposed in the double contours of the capillary wall (14). Moreover, presence of renal arteritis can be detected in renal biopsies of these patients, although, at least in our wide experience, no glomerular necrotizing extracapillary lesions have been found (13).

Materials and Methods

Renal Tissue

We selected for the study 25 renal biopsies of patients with ANCA-associated renal vasculitis and 18 cases of cryoGN, diagnosed by clinical, biochemical, and histologic means, including only cases with active lesions and no signs of glomerular sclerosis. Main clinical and histologic features are shown in Table 1. Normal kidney tissue was taken from 10 cadaver kidneys, not grafted because of vascular abnormalities.

Tissue samples for light microscopy, *in situ* hybridization, and *in situ* terminal DNA transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine stainings were performed according to standard techniques.

For immunofluorescence and immunoperoxidase staining, the un-fixed renal tissue was embedded in optimal cutting temperature (OCT) compound (Miles Scientific, Naperville, IL), snap-frozen in a mixture of isopentane and dry ice, and stored at -80°C . Subsequently, $5\text{-}\mu\text{m}$ sections were placed on slides and stored at -20°C until immunostained.

Immunoperoxidase Labeling

We used an avidin-biotin technique, as described previously (15), whereby a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. Briefly, after incubation with 0.5% avidin (Sigma Chimica, Gallarate, Milan, Italy) and 0.01% biotin (Sigma) to suppress endogenous avidin-binding activity, tissue sections were fixed in a methanol- H_2O_2 solution (to block endogenous peroxidase). After washing, sections were sequentially incubated with the primary antibody (see Table 2), with the secondary biotinylated antibody (Dako, Glostrup, Denmark) and with the peroxidase-labeled streptavidin (Dako). Peroxidase activity was detected with 3,5-diaminobenzidine (DAB; Dako), and then sections were counterstained with Harry's hematoxylin (BDH, Poole, England), dehydrated, and mounted in Entellan (Merck, Darmstadt, Germany). Specificity of labeling was demonstrated by the lack of staining after substituting phosphate-buffered saline (PBS) and proper control im-

Table 1. Main clinical and histologic features^a

Diagnosis	No. of Cases	Age at Renal Biopsy ^b	Clinical Features			Histologic Features			
			Serum Creatinine ($\mu\text{mol/L}$) ^b	C-ANCA (% of Cases)	P-ANCA (% of Cases)	Intracapillary Proliferation (0, +, ++, +++)	Necrotizing Extracapillary Lesions (% of Glomeruli)	Periglomerular Granulomas (% of Glomeruli)	Renal Arteritis (% of Cases)
CryoGN	18	55.4 ± 11.6	212.2 ± 167.9	—	—	+++ / ++++	0	0	11
WG	11	51.8 ± 14.7	229.8 ± 123.7	80	20	0	60 ± 15	18 ± 4	8
MPA	9	49.9 ± 10.4	229.8 ± 114.9	30	70	0	58 ± 20	15 ± 8	8
RLV	5	52.6 ± 11.9	203.3 ± 159.1	20	80	0	38 ± 29	10 ± 8	0

^a Values are mean ± SD. WG, Wegener's granulomatosis; MPA, microscopic polyarteritis; RLV, renal limited vasculitis; ns, not significant.

^b No significant differences between groups.

Table 2. Antibodies used for the study

Antibody	Specificity	Company
CD68	Monocyte-macrophages	Dako, Glostrup, Denmark
CD54	ICAM-1	Serotec, Kidlington, Oxford, UK
CD106	VCAM-1	Harlan Sera-Lab, Crawley Down, Sussex, UK
HLA class II	DP, DQ, DR antigens	Dako
27E10	MRP8/MRP14	Accurate, Westbury, NY
TNF- α	TNF- α	Genzyme, Cambridge, MA
IL-1 α	IL-1 α	Genzyme
PCNA	Proliferating cell nuclear antigen	Dako
Mib-1	Ki-67	Dako

TNF- α , tumor necrosis factor- α ; IL-1, interleukin-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

munoglobulins (Zymed, HistoLine, Milan, Italy) for the primary antibody.

Double Staining

According to the double-staining procedure published by Lan *et al.* (16), sections were treated as for the single stain from the beginning of the procedure to the DAB development, except for the use of Tris-buffered saline (TBS) instead of PBS. After washing, sections were then microwaved twice at 750 W for 5 min in citrate buffer. They were sequentially cooled, pretreated with levamisole, preincubated with normal serum, and incubated with the second primary antibody. After washing in TBS, sections were then labeled with a biotinylated secondary antibody (Dako) and alkaline phosphatase-conjugated streptavidin (Dako). Alkaline phosphatase activity was finally detected with Fast Red (Sigma). After sections were washed in distilled water and counterstained with Mayer's hematoxylin (Sigma), they were then mounted with an aqueous mounting medium (Ultamount, Dako). Specificity of labeling was demonstrated by the lack of staining after substituting TBS and irrelevant immunoglobulins for the first and/or second primary antibody.

In Situ Hybridization

Following the method published by Yamawaki *et al.* (17), we deparaffinized and rehydrated the tissues, then permeabilized them with saponin-TBS. Postfixation was carried out with 4% buffered paraformaldehyde. Sections were then acetylated with a solution of acetic-anhydride/triethanolamine. After washing, a prehybridization solution, containing SSC 10X, Denhardt's solution 10X, formamide 50%, Dextran sulfate 10%, and salmon sperm DNA 200 $\mu\text{g}/\text{ml}$ (all from Sigma), was applied for 1 h. Oligonucleotide digoxigenin-labeled probes (oligonucleotide cocktails specific for VCAM-1, IL-1, and TNF- α ; all from R&D Systems, Abingdon, UK) were appropriately diluted in the prehybridization solution and applied overnight in a water-saturated atmosphere. After hybridization, stringency washes were done with SSC 4X, SSC 2X – 50% formamide at 45°C, and again with SSC 1X at room temperature for 30 min. Specimens were pretreated with levamisole, washed in buffer, and covered with anti-digoxigenin antibody alkaline-phosphatase-conjugate (Boehringer Mannheim, Mannheim, Germany) for 3 h. The enzymatic reaction was developed with Fast Red. After sections were washed in distilled water, they were mounted with an aqueous mounting medium (Ultamount).

Positive controls were done using digoxigenin-labeled oligonucleotides for housekeeping genes (β -actin and G6PDH) and oligo-dT (all from R&D Systems). Negative controls were made using irrelevant

oligonucleotides, *i.e.*, digoxigenin-labeled oligonucleotide for rat insulin, (R&D Systems) and substituting the prehybridization solution for the probe solution.

Combined TUNEL and Immunohistochemistry

A standard TUNEL method (18) was applied with some modification. Briefly, tissues were deparaffinized and rehydrated. After suppression of endogenous avidin-binding activity, sections were treated with Proteinase K (Sigma), then endogenous peroxidase was blocked by 3% H_2O_2 . After washing, sections were covered with the appropriate labeling buffer (Clontech, M-Medical, Firenze, Italy). On each section, a solution of TdT enzyme and biotinylated dNTP mix with the addition of Mn^{++} , prepared following manufacturer's instructions (Clontech), was then applied. After treatment with stop buffer (Clontech), peroxidase-labeled streptavidin (Dako) was applied. Peroxidase activity was detected with DAB. After washing, sections were again microwaved in citrate buffer. They were sequentially cooled, pretreated with levamisole, preincubated with normal serum, and incubated with the primary antibody. Sections were then incubated with a biotinylated secondary antibody (Dako) and alkaline phosphatase-conjugated-streptavidin (Dako). Alkaline phosphatase activity was detected with Fast Red. Specificity of TUNEL reaction was tested by positive (pretreatment of separate slides with DNase; Sigma) and negative controls (substitution of an irrelevant protein for TdT enzyme).

Quantitative Evaluation

All immunostained sections were evaluated by an electronic image analysis system (ETC3000, Graftek, Villanterio, Pavia, Italy). Images were digitized using a videocamera (Kappa CF15/2, Gleichen, Germany) connected to a Leitz Diaplan microscope (Leica, Milan, Italy) and to a Pentium 120 computer (Maxwel, Rozzano, Italy) equipped with a frame grabber (Neotech Ltd, Eastleigh Hampshire, UK). An automated macro composed by a color threshold procedure, filtering and Danielsson algorithm, was applied on all digitized images. Cell count was performed considering the number of positive cells per glomerulus or per glomerulomatous lesion (200 \times). Glomerular analysis was performed after drawing a precise line along the Bowman's capsule. The electronic system was then programmed for region-of-interest analysis. A mean of 9 ± 2.3 (minimum, 6; maximum, 15) glomeruli/biopsy were analyzed. In periglomerular granulomas, it was impossible to discriminate between intraglomerular and periglomerular infiltration, but a limit to the periglomerular area was done assuming as standard area of evaluation the digitized image of the granu-

loma taken at the microscopic enlargement of 200× and corresponding to 76800 μm (2). Results were automatically exported and elaborated in an electronic file (Microsoft Excel 4.0) using the mean and the SD of the mean.

Statistical Analysis

Statistical significances ($P < 0.05$) were analyzed using the χ^2 test.

Results

The results of the quantitation of monocyte-macrophage infiltration are summarized in Table 3.

Normal Kidney

Rare monocyte-macrophages were present at the glomerular level (0.9 ± 0.4 CD68-positive cells/glomerulus). ICAM-1 was positive on glomerular endothelium. VCAM-1, completely negative in the glomerular tuft, was positive only on

Bowman’s capsule. *In situ* hybridization for VCAM-1 was completely negative in the glomerular tuft.

A complete negativity or a mild mesangioparietal staining was observed in normal glomeruli for HLA class II antigens. 27E10 antigen was completely negative. Whole glomerular negativity was also observed, by immunohistochemistry as well as by *in situ* hybridization, for TNF- α and IL-1 α .

No glomerular cells were positive for proliferation markers proliferating cell nuclear antigen (PCNA) and Mib-1. No cells were stained by TUNEL reaction.

ANCA-Positive Renal Vasculitis

Glomerular monocyte-macrophages were 44.8 ± 28.2 per glomerulus, almost exclusively accumulated in areas of necrotizing extracapillary lesions. Their presence was massive in periglomerular granulomatous reactions (163 ± 82.4 cells/granuloma) (Figure 1, a and b).

Table 3. Monocyte-macrophage infiltration^a

Antibodies	NK	ANCA + RV			P Values		
		Glomeruli	Periglomerular Granulomas	CryoGN	ANCA + RV vs NK	ANCA + RV (glomeruli) vs CryoGN	CryoGN vs NK
Immunohistochemistry							
CD68	0.9 ± 0.4 (0–2)	44.8 ± 28.2 (10.2–83.4)	163 ± 82.4 (72.7–256.4)	64.8 ± 12.3 (48.2–86.4)	0.001	ns	0.001
VCAM-1	0	66.4 ± 15.2 (32.8–89.6)	126.4 ± 58 (62.2–196.5)	0.03 ± 0.004 (0–2.1)	<0.001	<0.001	ns
HLA class II	0	59.6 ± 10.8 (38.9–86.2)	158.1 ± 60 (87.7–220.3)	10 ± 2.1 (4.3–15.6)	<0.001	0.02	<0.001
27E10	0	62.8 ± 12.2 (34.3–98.4)	198.2 ± 20.8 (150–260.8)	19.8 ± 2.4 (10.5–24.2)	<0.001	0.03	<0.001
TNF- α	0	46.7 ± 10.8 (27.3–68.9)	142.6 ± 68.9 (45.4–200.2)	8.2 ± 1.3 (3.1–12.7)	<0.001	0.01	<0.001
IL-1 α	0	39.5 ± 6.2 (20.7–54.2)	138.1 ± 62.8 (42.3–198.6)	7.4 ± 0.9 (3.9–10.6)	<0.001	0.01	<0.001
PCNA/CD68	0	5 ± 0.4 (0.6–8)	13.2 ± 0.6 (10.5–16.2)	0	0.01	0.01	ns
Mib-1/CD68	0	2.04 ± 0.3 (0.4–4.6)	4.7 ± 2.1 (1.2–8.8)	0	0.02	0.02	ns
<i>In situ</i> hybridization							
TUNEL/CD68	0	1.6 ± 0.7 (0.6–2.3)	3.2 ± 0.4 (1.8–4.7)	0	0.01	0.01	ns
mRNA							
VCAM-1	0	54.9 ± 10.1 (38.6–71.3)	106.2 ± 58.6 (49.6–176.2)	0	<0.001	<0.001	ns
TNF- α	0	40.6 ± 9.4 (22.2–60.6)	110.3 ± 54.2 (19.4–186.2)	7.8 ± 2.3 (2.9–14.6)	<0.001	0.01	<0.001
IL-1 α	0	31.2 ± 19.8 (4.9–64.2)	122.2 ± 63.7 (20.1–196.4)	6.4 ± 1.9 (1.8–10.9)	<0.001	0.01	<0.001

^a The number of positive cells/glomerulus is expressed as mean \pm SD of the mean. Minimum and maximum values are in parentheses. NK, normal kidney; ANCA + RV, antineutrophil cytoplasmic antibody–positive renal vasculitis; CryoGN, cryoglobulinemic glomerulonephritis.

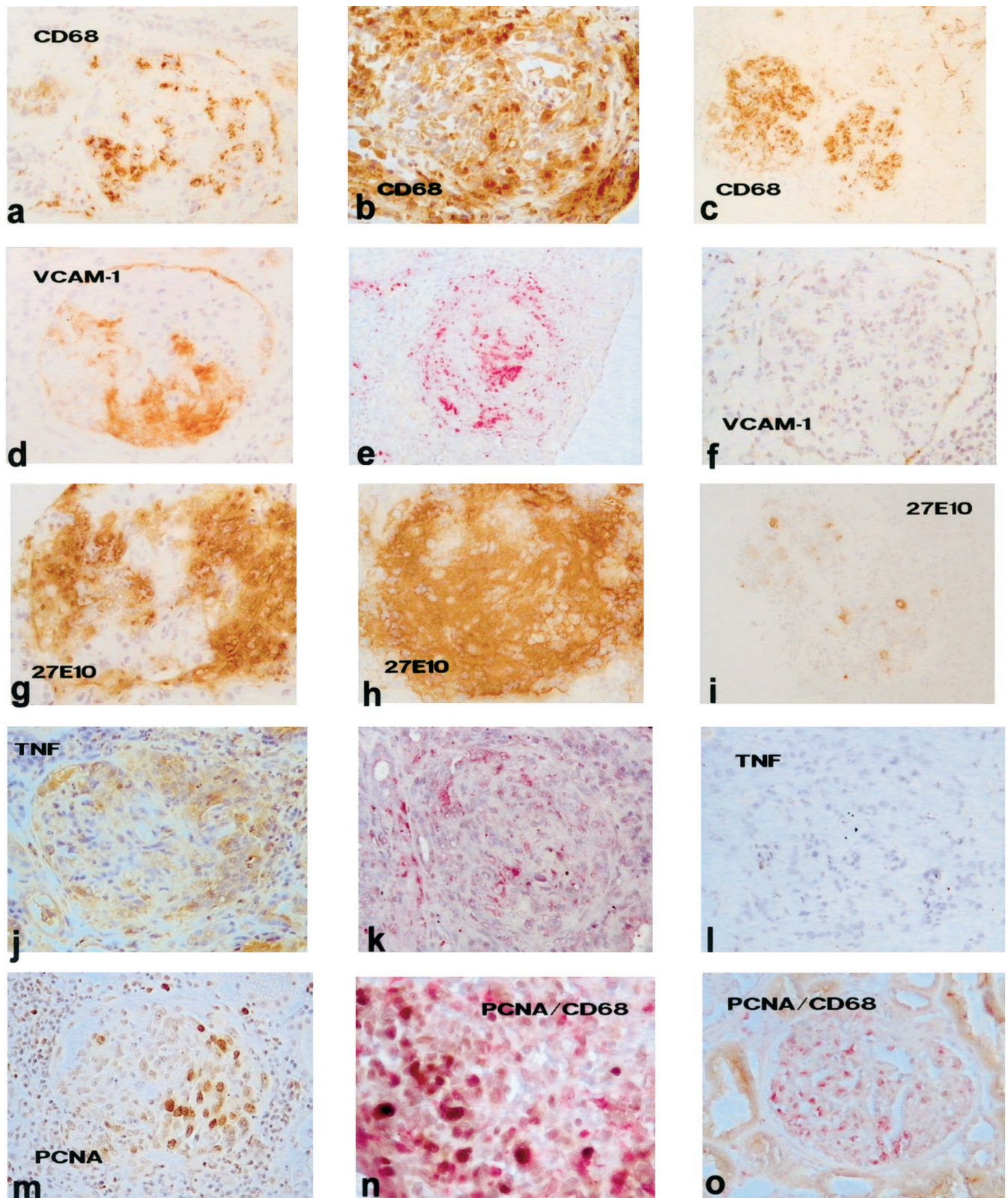


Figure 1. (a) Antineutrophil cytoplasmic antibody–positive renal vasculitis (ANCA+RV): The accumulation of a great number of macrophages (CD68-positive cells) is evident in a glomerulus, where they especially localize in an area of extracapillary proliferation (IPX, 200 \times). (b) ANCA+RV: An enormous amount of macrophages are present in a glomerular granulomatous lesion (IPX, 200 \times). (c) Cryoglobulinemic glomerulonephritis (cryoGN): Both glomeruli present a huge infiltration of macrophages, homogeneously occupying the glomerular tuft (IPX, 100 \times). (d) ANCA+RV: The adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) is clearly positive not only on Bowman's capsule but also in a well-delineated area of the tuft (IPX, 200 \times). (e) ANCA+RV: VCAM-1 mRNA demonstrated by *in situ* hybridization is specifically localized in an area of glomerular lesion. The positivity involves not only intraglomerular but also periglomerular infiltrating cells

ICAM-1 glomerular positivity was intense and diffuse to the whole tuft in all glomeruli. VCAM-1 specifically stained the areas of necrotizing extracapillary damage, labeling 66.4 ± 15.2 cells/glomerulus (Figure 1d). 126.4 ± 58 VCAM-1-positive cells were found in periglomerular granulomatous reactions. VCAM-1 mRNA was detected in the same glomerular areas, where it stained 54.9 ± 10.1 cells/glomerulus (Figure 1e). In granulomatous lesions, 106.2 ± 58.6 cells were positive.

A strong expression of HLA class II antigens was localized in areas of necrotizing extracapillary lesion (59.6 ± 10.8 cells/glomerulus) and in granulomatous reactions, where 158.4 ± 60.1 cells were labeled. 27E10 antigen was intensely positive in areas of necrotizing extracapillary damage (62.8 ± 12.2 cells/glom), and its expression was massive in periglomerular granulomas (198.2 ± 20.8 cells/granuloma) (Figure 1, g and h).

TNF- α and IL-1 α staining were similar in both intensity and distribution: glomerular staining was detected in areas of necrotizing extracapillary damage (46.7 ± 10.8 TNF-positive cells/glomerulus; 39.5 ± 6.2 IL-1-positive cells/glomerulus) and in granulomatous reactions (142.6 ± 68.9 TNF-positive cells/granuloma; 138.1 ± 62.8 IL-1-positive cells/granuloma). *In situ* hybridization paralleled immunohistochemistry (40.6 ± 9.4 TNF-positive cells/glomerulus; 31.2 ± 19.8 IL-1-positive cells/glomerulus; 110.3 ± 54.2 TNF-positive cells/granuloma; 122.2 ± 63.7 IL-1-positive cells/granuloma) (Figure 1, j and k).

PCNA was positive on 14 ± 2 cells/glomerulus (Figure 1m) and 38.3 ± 12.2 cells/granuloma. Of them, 5 ± 0.4 cells/glomerulus and 13.2 ± 0.6 cells/granuloma were double stained for PCNA and CD68 antigen (Figure 1n). Mib-1 staining was present on 6.7 ± 2.3 cells/glomerulus and 18.8 ± 7.9 cells/granuloma. By double staining, 2.04 ± 0.3 cells/glomerulus and 4.7 ± 2.1 cells/granuloma also were found to be CD68 positive.

With the use of the TUNEL reaction coupled with immunohistochemistry, it was possible to recognize some CD68-positive cells that were also positive for TUNEL reaction (1.6 ± 0.7 cells/glomerulus; 3.2 ± 0.4 cells/granuloma).

The variability of all markers among different specimens is given in Table 3 (values in parentheses). Considering singularly every specimen, glomeruli without necrotizing extracapillary lesions appeared almost normal also at the immunohistochemical and *in situ* hybridization evaluation. No statistical significance was reached comparing cases divided by ANCA pattern or by diagnosis.

Cryoglobulinemic Nephritis

Glomerular macrophages were present in very high numbers (64.8 ± 12.3 cells/glomerulus), homogeneously occupying the entire glomerular tuft (Figure 1c). No granulomatous lesions but rare periglomerular macrophages were found (2.6 ± 0.4).

ICAM-1 positivity was strong and diffuse in all glomeruli. Conversely, VCAM-1 was completely negative or showed only a scattered cellular positivity (0.03 ± 0.004 cells/glomerulus) (Figure 1f). VCAM-1 mRNA was not detected.

Expression of HLA class II antigen was found on scattered glomerular cells (10 ± 2.1 cells/glomerulus). 27E10 antigen was present on 19.8 ± 2.4 cells/glomerulus (Figure 1i). Scattered positivity was also found for TNF- α (8.2 ± 1.3 cells/glomerulus) and IL-1 α (7.4 ± 0.9 cells/glomerulus), both the protein and the mRNA (Figure 1l).

PCNA was positive on 0.7 ± 0.1 cells/glomerulus, and Mib-1 was detected very rarely on cells (0.09 ± 0.04). Double staining did not show monocyte-macrophages positive for both proliferation markers (Figure 1o). No macrophages were double-stained by CD68 marker and TUNEL reaction.

The variability of all markers among different specimens is given in Table 3 (values in parentheses). Almost no variability was found among different glomeruli in the same specimen.

Discussion

Our results confirm that large numbers of glomerular macrophages are present in renal vasculitis as well as in cryoGN, although their distribution seems profoundly different. In fact, in renal vasculitis, we found them strictly co-localized with areas of necrotizing extracapillary lesion and even more numerous in periglomerular granulomas. In cryoglobulinemia, macrophages seemed to be homogeneously distributed in the glomerular tuft and no signs of relevant periglomerular infiltration could be found. This difference, obviously linked to the distinct pathogenesis of glomerular lesions, was also reflected in a different expression of adhesion molecules.

Among Ig-like adhesion molecules, ICAM-1 is expressed by glomerular endothelium in normal kidneys and has been found to be upregulated in most glomerulonephritis (7,19,20). Also in our cases, independent of the disease, ICAM-1 upregulation was present in all glomeruli examined.

Conversely, we detected VCAM-1 glomerular positivity only in renal vasculitis and only in damaged glomeruli, where the molecule clearly stained the areas of necrotizing extracapillary lesions, leaving the remaining tuft negative. This is

(alkaline phosphatase [AP], 100 \times). (f) CryoGN: VCAM-1 is positive only on Bowman's capsule and leaves all of the tuft completely negative (IPX, 200 \times). (g) ANCA+RV: The antigen 27E10 is markedly positive in a damaged glomerulus and many periglomerular cells (IPX, 200 \times). (h) ANCA+RV: The same marker of acute activation (27E10) shows massive positivity in a glomerular granulomatous lesion (IPX, 200 \times). (i) CryoGN: The same two glomeruli in panel c possess only scattered and faint positivity for the 27E10 antigen (IPX, 100 \times). (j) ANCA+RV: A circumferential extracapillary proliferation is strongly positive for the antibody anti-tumor necrosis factor- α (anti-TNF α) (IPX, 200 \times). (k) ANCA+RV: By *in situ* hybridization, many glomerular cells that are positive for TNF- α mRNA are detectable (AP, hematoxylin counterstain, 200 \times). (l) CryoGN: The complete glomerular negativity for TNF antibody is clearly evident (IPX, 200 \times). (m) ANCA+RV: In a segmental crescent, most cells are proliferating cell nuclear antigen (PCNA) positive (IPX, 200 \times). (n) ANCA+RV: In a glomerular granulomatous lesion, several cells are double-stained by PCNA (in brown) and CD68 (in red), revealing macrophages entering the cell cycle (IPX/AP, 400 \times). (o) CryoGN: A great number of glomerular macrophages (in red) are not stained by PCNA (in brown) (IPX/AP, 200 \times).

especially relevant because it is a *de novo* production, confirmed by VCAM-1 mRNA positivity in the same areas. A similar expression of VCAM-1 has been observed by our group (1,21) in other forms of glomerular capillaritis, *i.e.*, necrotizing IgA nephritis, Henoch-Schönlein syndrome, and glomerulonephritis associated with endocarditis, and never found in other glomerular diseases, suggesting that necrosis of glomerular capillaries and VCAM-1 production are strictly related events and that in these lesions VCAM-1 is fundamental in monocyte adhesion.

Moreover, in renal vasculitis, VCAM-1 seems to be expressed also by macrophages, not only in the glomerular tuft but also in periglomerular granulomatous lesions. Detection of VCAM-1 mRNA in these cells cannot exclude that soluble VCAM-1 links to its integrin receptor very late activation-4 on macrophage surface, as proposed by Ogawa *et al.* (22), but certainly confirms our previous results (23) and results obtained by other investigators (24,25). A possible explanation is related to macrophage activation and consequent production of adhesion molecules by themselves. To our knowledge, there are no culture studies demonstrating production of VCAM-1 by macrophages, but, for instance, ICAM-1 production by eosinophils after cytokine stimulation has been demonstrated by Czech *et al.* (26). Moreover, isolation from human synovium of macrophages expressing VCAM-1 has been performed by Koch *et al.* (27) in patients with rheumatoid arthritis. Although their significance is not completely clarified, expression of Ig-like adhesion molecules by leukocytes can be important in cell–cell and cell–matrix interaction, contributing to overall tissue inflammation.

Acute activation of macrophages seems to occur in both renal vasculitis and cryoGN, whereas normal kidney resident macrophages did not show activation markers. Cell culture studies show that acute macrophage activation is followed by increase in expression of HLA class II molecules on the cell surface (28). Although we have found a strong expression of HLA class II antigens in areas of macrophage accumulation, statistically different not only from normal kidneys but also from cryoGN, demonstration of a cellular increase is difficult in the tissue. Instead, a better marker for acute activation can be the so-called 27E10 antigen (29). 27E10 is a dimer of two calcium-binding proteins, MRP8 and MRP14, monomeric and intracytoplasmic in normal conditions. Formation of the dimer and expression on the cell surface occur only after acute activation. The molecule disappears from the cell surface when the macrophage proceeds in its further steps of maturation and has been found completely absent from tissues with chronic inflammation (30). Our data show that in glomerular lesions in renal vasculitis, almost all macrophages are simultaneously acutely activated, whereas in cryoglobulinemic nephritis, only approximately one third of glomerular macrophages are 27E10-positive cells and in normal kidneys the molecule is completely absent. Moreover, a massive 27E10 expression characterizes periglomerular granulomas, differentiating these peculiar and acute lesions from other kinds of tissue granulomas, in which the molecule has been found completely negative (30).

Recent experimental models have demonstrated that TNF- α and IL-1 production strictly depends on macrophage infiltra-

tion (31,32). Moreover, in cell separation experiments, it has been demonstrated that macrophages that express 27E10 epitope produce the greatest quantities of TNF- α and IL-1 (30). Our data seem to confirm these results; in fact, TNF- α and IL-1 expression, in terms of protein as well as mRNA, exactly paralleled 27E10 staining. A stronger expression of both cytokines has been detected in renal vasculitis in comparison with cryoGN, despite a similar number of macrophages, suggesting that acute activation directly influences the quantity of proinflammatory products in the tissue, making the disease more severe. TNF- α and IL-1 possess and share a large spectrum of proinflammatory activities, and new promising treatments can be based on their inhibition (33). Recent research has shown, in fact, that inhibition of IL-1, through IL-1 receptor antagonist administration, is able not only to prevent the development of anti-glomerular basement membrane nephritis in rats (34) but also to induce, by late administration, regression of the disease (35), and similar effects have been obtained by blocking TNF- α by administration of a soluble form of its receptor (34,36).

In renal vasculitis but not in cryoGN or normal kidneys, we found glomerular and periglomerular macrophages positive for proliferation markers. Similar results have recently been obtained in experimental models, where proliferating macrophages have been found, for instance, in anti-glomerular basement membrane nephritis but not in normal resident macrophages or in a model of hypercholesterolemic glomerulonephritis (36). In agreement with those authors, we suggest that local macrophage proliferation can be an important mechanism of damage amplification, but further follow-up studies are required to understand better whether in human diseases the presence or absence of macrophage proliferation is also associated with a different prognosis.

Given the intense accumulation of macrophages, as a result not only of recruitment but also of proliferation, our results that the presence of macrophage apoptosis was found only in renal vasculitis are not surprising. Apoptosis could in fact be relevant for macrophage clearance from the kidney, counterbalancing their overaccumulation, and could be an important regulatory mechanism of progression of renal damage, as also suggested by Lan *et al.* (37).

Taken together, our data seem to clarify some properties of macrophage infiltration in renal vasculitis, especially in comparison with cryoGN. In fact, despite their comparable number in glomerular areas of accumulation, macrophages show qualitative differences in terms of glomerular localization, VCAM-1-mediated adhesion, proliferation, and apoptosis. Moreover, acute activation and production of proinflammatory products seem to be present at the same time in a greater number of macrophages in renal vasculitis, giving to this disease a stronger severity that could be taken into account for therapeutic strategies.

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