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# Cytotoxic effects of antibodies to proteinase 3 (C-ANCA) on human endothelial cells

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## SUMMARY

Autoantibodies directed against cytoplasmic antigens of neutrophils (ANCA), especially those with specificity for proteinase 3 (PR-3) and myeloperoxidase, are valuable markers for differential diagnosis and monitoring of disease activity in Wegener's granulomatosis (WG) and other vasculitides. Till now, several concepts concerning a direct role of antibodies against PR-3 in the pathogenesis of WG have been discussed. Recently we were able to show that these antibodies recognize PR-3 translocated into the membrane of human endothelial cells. The aim of this study was to investigate putative cytotoxic effects of antibodies to PR-3 on human endothelial cells. Antibodies were obtained by affinity purification of sera from patients with active WG. Purified antibodies to Ro (SS-A), La (SS-B) and RNP served as controls. Purified antibodies to PR-3 displayed a lytic activity against endothelial cells treated with tumour necrosis factor-alpha (TNF-$\alpha$) with the help of cytokine-primed neutrophils as measured in a Cr-release assay. About 100% specific cytotoxicity occurred after 4 h and was independent of complement. Cytotoxic effects were inhibited by coincubation with unprimed neutrophils or preincubation of PR-3 antibodies with purified antigen. Antibodies to Ro (SS-A), La (SS-B) or RNP had no cytotoxic effect. In summary, PR-3 antibody-induced cytotoxicity required (i) expression of PR-3 on the surface of TNF-$\alpha$-treated endothelial cells; and (ii) co-cultivation of cytokine-primed neutrophils. This is to the best of our knowledge the first report on direct cytotoxic effects of PR-3 antibodies on vascular endothelium. Our data give a hint at a PR-3 antibody-mediated mechanism of endothelial injury via antibody-dependent cellular cytotoxicity in WG and other ANCA-related vasculitides.

**Keywords** ANCA proteinase 3 cytotoxicity endothelial cells vasculitis

## INTRODUCTION

Circulating autoantibodies directed against neutrophil cytoplasmic antigens (ANCA), especially those with specificity for proteinase 3 (PR-3) or myeloperoxidase (MPO), are specific markers for systemic vasculitides such as Wegener's granulomatosis (WG) and microscopic polyarteritis [1,2]. They have been proposed to have a pathogenic role in vasculitis due to the correlation of ANCA with these diseases [3]. Since endothelium forms the barrier between the circulation and surrounding tissue, binding of antibodies to endothelial surface antigens is potentially significant in the pathogenesis of vascular damage, leading to altered permeability and exacerbation of the disease.

Recently we were able to show that tumour necrosis factor-alpha (TNF-$\alpha$), IL-1$\alpha/\beta$ and interferon-gamma (IFN-$\gamma$) led to an increased PR-3 expression in the cytoplasm of human endothelial cells (HEC) by performing polymerase chain reaction (PCR) analysis, Western blot, cyto-ELISA and laser scanning microscopy. In addition we were able to demonstrate that PR-3 was also translocated into the cell membrane, thereby becoming accessible to ANCA [4].

Until now several concepts concerning a direct role of anti-PR-3 antibodies in the pathogenesis of WG have been discussed in literature, and interaction of ANCA with HEC may play a key role [5]. The aim of the present study was to investigate putative cytotoxic effects of anti-PR-3 antibodies on HEC.

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## MATERIALS AND METHODS

*Serum samples*

Serum samples were obtained from 185 donors. Fifty suffered from WG. The diagnosis was established on the basis of classic symptoms and the typical histological findings in biopsy specimens as described earlier [6]. Sera of 100 healthy blood donors (HBD), 25 patients with systemic lupus erythematosus (SLE), five patients with Sharp syndrome, and five patients with Sjögren's syndrome (SS) served as controls.

*Antibody testing*
All WG sera were tested for anti-PR-3 antibodies by immunofluorescence technique (IFT) on fixed neutrophils [1], ELISA and Western blot. Several antigen preparations served as antigens: alpha extract of human neutrophils [7], purified PR-3 [8], MPO, cathepsin G and elastase [9]. ELISAs were performed as described earlier [10]. PAGE and Western blotting were performed as described previously [11,12]. In addition, all sera were screened with routine methods for other antibody specificities [11].

*Purification of anti-PR-3 antibodies*
IgG was prepared from five monospecific anti-PR-3 antibody-positive WG sera (WG 1, 3, 4: patients clinically active; WG 2, 3: patients in remission; C-ANCA-titres 1:80–1:640) by ammonium sulphate precipitation and ion exchange chromatography on DEAE-Sephadex (Pharmacia, Uppsala, Sweden). Anti-PR-3 antibodies were affinity-purified as described earlier [13] using purified PR-3. $F(ab')_2$ fragments were prepared as previously described [6]. For inhibition experiments affinity-purified anti-PR-3 antibodies were mixed with different extracts of human neutrophils, an extract of HEp2 cells (control), affinity-purified PR-3 antigen and PR-3 purified as described by Kao *et al.* [8] v/v diluted to 0·1 mg/ml protein concentration in PBS and incubated on a rotator for 1 h at 37°C and 12 h at 4°C. The mixture was centrifuged at 30 000 ***g*** for 15 min at 4°C and the supernatants were kept as absorbed material. An anti-PR-3-specific B cell clone was established and characterized earlier [14]. Antibody preparations were tested for endotoxin using a commercially available E-toxate assay (Sigma, Deisenhofen, Germany).

*Purification of anti-Ro (SS-A), anti-La (SS-B) and anti-RNP antibodies*
Antibodies to Ro (SS-A), La (SS-B) and RNP were purified and characterized as described previously [13].

*Preparation of cell extracts*
An extract (alpha-fraction) of human neutrophils was prepared as described by Rasmussen *et al.* [7] and Savage *et al.* ('acid' extract) [15]. An extract of HEp2 cells was prepared as described earlier [6]. Total extracts of cytokine-treated HEC were prepared according to Albeda *et al.* [16]. Only cell cultures free of fibroblasts or monocytes obtained after several passages were used for these experiments.

*Purification of PR-3*
PR-3 was purified as described by Kao *et al.* [8] and affinity-purified as described by Lüdemann *et al.* [12] using an extract of granulocytes.

*Isolation and culture of human endothelium cells*
Human umbilical cord endothelial cells (HEC) were isolated according to the method of Jaffe *et al.* [17] and cultured under standard conditions [18,19]. These cells were used for further experiments between passages 4 and 6. Cells of 10 donors were pooled to exclude the influence of blood group antigens. Morphology was confirmed by phase contrast light microscopy showing the typical cobblestone monolayer appearance of cells. Purity of culture was tested with antibodies to factor VIII antigen and Ulex lectine [14]. HEC were passaged on gelatine-coated culture slides (Lab-Tek; Miles Scientific, Naperville, IL) or Primaria culture dishes (Falcon, NJ, USA) and fixed with ethanol 96%, methanol (abs) or paraformaldehyde 3·7% (−20°C for 10 min) [12]. TNF-$\alpha$ (3 ng/ml; Boehringer, Mannheim, Germany) was added to the medium before fixation to induce membrane expression of PR-3.

*Isolation of neutrophils*
Neutrophils were isolated as recently described by Toothill *et al.* [20].

*Cytotoxicity assay*
HEC were grown in 96-well plates (Greiner no. 650180, Nuertingen, Germany) until forming a confluent monolayer. Unfixed cells (untreated or pretreated with TNF-$\alpha$ (1 ng/ml, 2 h)) were incubated with 1 $\mu$Ci $^{51}$Cr (1 mCi/ml) in 100 $\mu$l culture medium for 18 h at 37°C. Each step was carefully monitored using phase contrast microscopy regarding detection of the monolayer or change of cell morphology. During the test no such effects could be seen. Cells were washed three times with culture medium to remove excess $^{51}$Cr.

Respective antibody samples (affinity purified; (2–0·1 mg/ml IgG) 5× anti-PR-3, 1× anti-dsDNA, 2× anti-Ro, 2× anti-La, anti-endothelial-leucocyte adhesion molecule-1 (ELAM-1), pool HBD) in culture medium (100 $\mu$l) were added to each well, and tests were performed in triplicate. In some tests 25 $\mu$l of fresh rabbit complement (kindly donated by Professor B. Fleischer, B. Nocht Institute, Hamburg, Germany) were added.

All antibody probes were tested under the following conditions: (i) HEC without TNF-$\alpha$ treatment, no complement; (ii) HEC without TNF-$\alpha$ treatment, with complement; (iii) HEC with TNF-$\alpha$, no complement; (iv) HEC with TNF-$\alpha$, with complement.

Plates were centrifuged at 50 ***g*** for 5 min and free radioactivity of 50 $\mu$l supernatant was measured in a gamma counter (Cobra; Canberra Packard, Frankfurt, Germany). Free activity in the supernatant was measured as spontaneous lysis. Free activity was measured after maximal lysis by addition of 100 $\mu$l 0·1% SDS-0·025 M NaOH. Specific lysis was calculated as

$$\frac{\text{ct/min test} - \text{ct/min spontaneous}}{\text{ct/min total} - \text{ct/min spontaneous}}$$

To investigate antibody-dependent cellular cytotoxicity (ADCC), cytotoxicity tests were performed as described above. In addition, some wells were preincubated with $2{\cdot}5 \times 10^4$ neutrophils preactivated with 400 U/ml IFN-$\gamma$ for 10 h [21] to induce maximal expression of FcRI and FcRII receptors. Before incubation with neutrophils, excess anti-PR-3 antibodies were washed away.

Purified anti-PR-3 antibodies, antibodies to dsDNA, Ro, La and a pool of HBD were tested on: (i) HEC without TNF-$\alpha$ or complement, but in the presence of preactivated neutrophils; (ii) HEC without TNF-$\alpha$ in the presence of complement and preactivated neutrophils; (iii) HEC pretreated with TNF-$\alpha$ (1 ng/ml) for 2 h without complement, but in the presence of preactivated neutrophils; (iv) HEC pretreated with TNF-$\alpha$ (1 ng/ml) for 2 h in the presence of complement and preactivated neutrophils; (v) human fibroblasts (CRL1905 - CCD

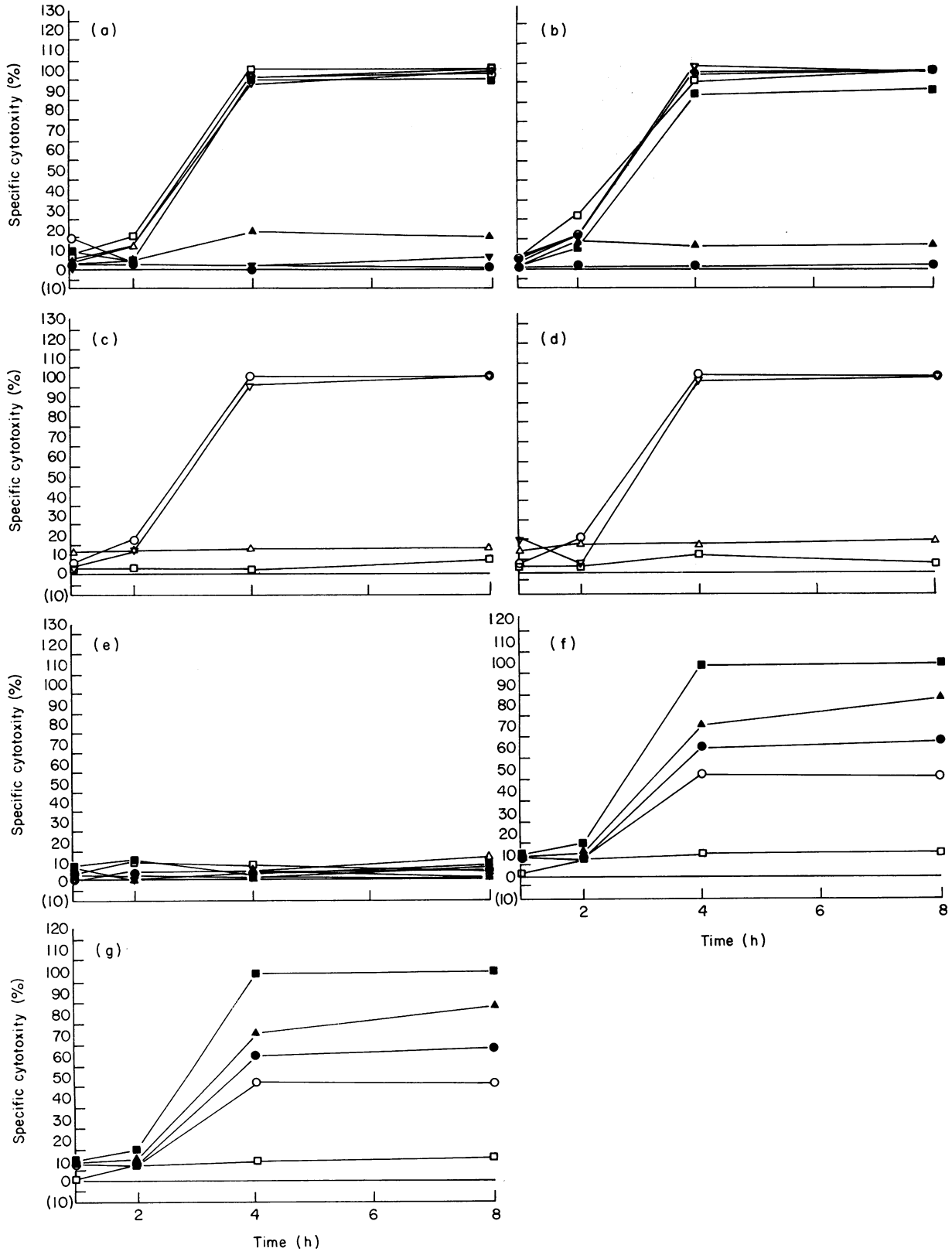
(a)
(b)
(c)
(d)
(e)
(f)
(g)
Specific cytotoxity (%)
Time (h)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
(10)
2
4
6
8

**Table 1.** Data of Fig. 1a and e

| Time (h) | WG1 | WG2 | WG3 | WG4 | WG5 | dsDNA | Ro1 | Ro2 | La1 | La2 | HBD | Neutr. only | Unpr. neutr. | Spont. | Max. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2149·3 | 2138·7 | 2867·6 | 2238·6 | 2156·7 | 1877·9 | 1831·4 | 1766·3 | 2479·3 | 2639·7 | 1938·4 | 1968·3 | 2064·3 | 1764·3 | 7464·3 | CT |
| | 2696·6 | 1903·8 | 3059·7 | 2475·6 | 2693·8 | 2146·3 | 2096·3 | 1938·4 | 2388·3 | 2183·4 | 2267·6 | 1593·6 | 2111·5 | 1957·8 | 7569·4 | CT |
| | 2238·4 | 2269·3 | 2468·9 | 1906·3 | 2076·5 | 2278·9 | 2147·5 | 2163·9 | 2174·6 | 1976·6 | 2074·9 | 1686·7 | 2088·0 | 2036·7 | 7653·3 | CT |
| | 2361·4 | 2103·9 | 2798·7 | 2206·8 | 2425·3 | 2101·0 | 2025·1 | 1956·2 | 2347·4 | 2266·6 | 2093·6 | 1789·5 | 2087·9 | 1919·6 | 7567·7 | M |
| | 293·7 | 185·2 | 301·4 | 285·9 | 379·8 | 204·3 | 169·7 | 199·4 | 156·4 | 339·3 | 165·4 | 195·0 | 23·6 | 140·1 | 86·5 | s.d. |
| | 7·8 | 3·3 | 15·6 | 5·1 | 8·9 | 3·2 | 1·9 | 0 | 7·6 | 3·1 | 3·1 | 0 | 2·9 | | | CYT |
| 2 | 3067·3 | 2786·3 | 2133·6 | 2439·6 | 1839·4 | 2493·6 | 2536·3 | 2279·3 | 2306·7 | 1839·6 | 1739·1 | 1796·6 | 2149·0 | | | CT |
| | 2983·6 | 2658·9 | 1896·3 | 2577·2 | 2014·3 | 2783·6 | 1899·4 | 2086·7 | 2653·9 | 1766·3 | 2467·3 | 1838·4 | 2284·3 | | | CT |
| | 2496·3 | 2179·6 | 2287·6 | 2691·4 | 2539·6 | 1969·7 | 1963·9 | 2116·7 | 2016·3 | 2036·7 | 1896·4 | 2033·6 | 2016·7 | | | CT |
| | 2849·0 | 2541·6 | 2105·8 | 2569·4 | 2131·1 | 2408·9 | 2133·2 | 2160·9 | 2480·3 | 1880·9 | 2034·4 | 1879·5 | 2150·0 | | | M |
| | 308·3 | 319·9 | 197·1 | 126·1 | 367·4 | 423·3 | 350·6 | 103·6 | 245·5 | 139·8 | 382·9 | 109·4 | 133·8 | | | s.d. |
| | 16·4 | 11·0 | 3·3 | 11·5 | 3·7 | 8·7 | 3·8 | 4·3 | 9·9 | 0 | 2·0 | 0 | 4·1 | | | CYT |
| 4 | 7649·6 | 7761·4 | 7136·6 | 7004·3 | 7786·0 | 2637·9 | 2283·6 | 1936·7 | 1988·5 | 1736·4 | 1637·6 | 1536·7 | 3086·0 | | | CT |
| | 7889·3 | 7203·9 | 7949·8 | 7283·6 | 7312·7 | 1864·3 | 2173·6 | 2286·3 | 2031·6 | 2137·8 | 1839·6 | 1603·8 | 2936·5 | | | CT |
| | 7653·0 | 7046·2 | 6938·7 | 7106·9 | 7039·4 | 2347·3 | 1967·8 | 2144·5 | 2056·7 | 2293·6 | 1967·8 | 2406·3 | 2866·7 | | | CT |
| | 7730·6 | 7337·2 | 7341·7 | 7131·6 | 7279·3 | 2284·5 | 2141·7 | 2122·5 | 2025·6 | 2055·9 | 1815·0 | 1848·9 | 2963·0 | | | M |
| | 137·4 | 375·8 | 535·8 | 141·3 | 225·1 | 388·6 | 160·3 | 175·8 | 34·5 | 287·5 | 166·5 | 438·8 | 112·0 | | | s.d. |
| | 102·9 | 95·9 | 95·9 | 92·3 | 94·9 | 6·5 | 3·9 | 3·6 | 1·9 | 2·4 | 0 | 0 | 18·4 | | | CYT |
| 8 | 8359·6 | 7853·8 | 7569·6 | 7826·3 | 7663·3 | 2298·6 | 2386·6 | 1896·7 | 2236·7 | 1836·6 | 2079·6 | 2074·9 | 3065·3 | | | CT |
| | 7569·7 | 7536·7 | 7238·2 | 7699·5 | 7196·6 | 1876·3 | 2537·1 | 1866·7 | 2383·4 | 2493·5 | 1860·3 | 2538·6 | 2689·0 | | | CT |
| | 7736·8 | 7658·3 | 7496·7 | 6947·8 | 6836·3 | 2109·8 | 2538·4 | 2143·9 | 2374·3 | 2116·3 | 1597·8 | 1897·6 | 2536·5 | | | CT |
| | 7888·7 | 7682·9 | 7434·8 | 7491·2 | 7232·0 | 2094·8 | 2487·3 | 1969·0 | 2331·5 | 2148·8 | 1847·8 | 2170·4 | 2763·8 | | | M |
| | 416·3 | 159·9 | 174·1 | 474·8 | 414·6 | 211·5 | 87·7 | 152·2 | 82·2 | 329·6 | 241·4 | 330·9 | 272·0 | | | s.d. |
| | 105·7 | 102·0 | 97·6 | 98·6 | 94·1 | 3·1 | 10·1 | 0 | 7·3 | 4·1 | 0 | 4·0 | 14·9 | | | CYT |

Spont, Spontaneous lysis; max; maximal lysis; CT, ct/min; M, mean; CYT, calculated specific cytotoxicity.

**Fig. 1.** (a) $^{51}$Cr-release assay with tumour necrosis factor-alpha (TNF-$\alpha$)-treated human endothelial cells (HEC) (coincubation with IFN-$\gamma$-treated neutrophils and complement). One mark represents mean of three measurements (data including s.d. are given in Table 1). WG1–5, affinity-purified anti-proteinase 3 (PR-3) antibodies (sera of five different Wegener's granulomatosis (WG) patients); maximum effect after 4 h; HBD, IgG preparation from a pool of healthy blood donors (HBD); no effect; Unpr. neutr., coincubation of affinity-purified anti-PR-3 antibodies (WG1) with neutrophils that were not pretreated (primed) with IFN-$\gamma$ (controls); no effect; Neutr. only, reaction after coincubation of IFN-$\gamma$-treated neutrophils (without anti-PR-3 antibodies); no effect. □, WG1; △, WG2; ○, WG3; ▽, WG4; ■, WG5; ▲, unprimed neutrophils; ▼, neutrophils only; ●, HBD. (b) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils, no complement). One mark represents mean of three measurements (data including s.d. are given in Table 2). WG1–5, affinity-purified anti-PR-3 antibodies (sera of five different WG patients); maximum effect after 4 h; Neutr. only, reaction after coincubation of IFN-$\gamma$-treated neutrophils (without anti-PR-3 antibodies); no effect; HBD, IgG preparation from a pool of healthy blood donors; no effect. □, △, etc., as in (a). (c) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils, no complement). One mark represents mean of three measurements. WG 2a (□), reaction of an affinity-purified anti-PR-3 antibody after preincubation with alpha extract of neutrophils; no reaction; WG 2b (△), reaction of an affinity-purified anti-PR-3 antibody after preincubation with purified PR-3 (Kao *et al.* [8]); no reaction; WG 2c (○), reaction of an affinity-purified anti-PR-3 antibody after preincubation with packed erythrocytes; maximum effect after 4 h; WG 2d (▽), reaction of an affinity-purified anti-PR-3 antibody after preincubation with an extract of HEp2 cells; maximum effect after 4 h. (d) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils, no complement). One mark represents mean of three measurements. WG 3a (□), reaction of an affinity-purified anti-PR-3 antibody after preincubation with alpha extract of neutrophils; no reaction; WG 3b (△), reaction of an affinity-purified anti-PR-3 antibody after preincubation with purified PR-3 (Kao *et al.* [8]); no reaction; WG 3c (○), reaction of an affinity-purified anti-PR-3 antibody after preincubation with packed erythrocytes; maximum effect after 4 h; WG 3d (▽), reaction of an affinity-purified anti-PR-3 antibody after preincubation with an extract of HEp2 cells; maximum effect after 4 h. (e) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils and complement). One mark represents mean of three measurements (data including s.d. are given in Table 1). dsDNA (□), Ro1 (△), Ro2 (○), La1 (■), La2 (●): affinity-purified antibodies (controls); no effect. HBD (▲), IgG preparation from a pool of healthy blood donors; no effect. Neutr. only (▽), reaction after coincubation of IFN-$\gamma$-treated neutrophils (without anti-PR-3 antibodies); no effect. (f) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils and complement). One mark represents mean of three measurements (data including s.d. are given in Table 3). WG1 (■, 2·0 mg/ml; ▲, 1·0 mg/ml; ●, 0·5 mg/ml; ○, 0·1 mg/ml), affinity-purified anti-PR-3 antibodies (IgG 2·0–0·1 mg/ml; serum of a clinically active WG patient); maximum effect after 4 h. Fibroblast (□), $^{51}$Cr-release assay as described (IgG 2·0 mg/ml), but human fibroblasts instead of HEC; no effect (control). (g) $^{51}$Cr-release assay with TNF-$\alpha$-treated HEC (coincubation with IFN-$\gamma$-treated neutrophils and complement). One mark represents mean of three measurements (data including s.d. are given in Table 3). WG2 (■, 2·0 mg/ml; ▲, 1·0 mg/ml; ●, 0·5 mg/ml; ○, 0·1 mg/ml), affinity-purified anti-PR-3 antibodies (IgG 2·0–0·1 mg/ml; serum of a WG patient in remission); maximum effect after 4 h. Fibroblast (□), $^{51}$Cr-release assay as described (IgG 2·0 mg/ml), but human fibroblasts instead of HEC; no effect (control).

**Table 2.** Data of Fig. 1b

| Time (h) | WG1 | WG2 | WG3 | WG4 | WG5 | HBD | Neutr. only | Unpr. neutr. | Spont. | Max. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2006·3 | 1937·8 | 2049·6 | 2477·8 | 1658·3 | 2015·6 | 2066·3 | 1939·3 | 1853·6 | 7356·9 | CT |
| | 2143·7 | 2493·5 | 2136·8 | 2139·6 | 1976·5 | 1538·5 | 1596·4 | 1857·5 | 1976·3 | 7849·7 | CT |
| | 2565·6 | 2267·9 | 1987·3 | 1847·3 | 2237·6 | 1531·6 | 1437·8 | 2039·6 | 1811·6 | 7957·8 | CT |
| | 2238·5 | 2233·1 | 2057·9 | 2154·9 | 1957·6 | 1695·2 | 1700·2 | 1945·5 | 1880·5 | 7721·5 | M |
| | 291·5 | 279·4 | 75·1 | 315·5 | 290·1 | 277·5 | 326·8 | 91·2 | 85·6 | 320·7 | s.d. |
| | 6·1 | 6·0 | 3·0 | 4·7 | 1·3 | 0 | 0 | 1·1 | | | CYT |
| 2 | 3596·4 | 2538·6 | 2649·6 | 2279·6 | 1839·7 | 1839·6 | 1736·2 | 2643·5 | | | CT |
| | 3847·9 | 2879·5 | 2538·7 | 2938·9 | 2533·6 | 1943·8 | 1598·6 | 2573·8 | | | CT |
| | 2938·6 | 3169·5 | 3287·0 | 3397·6 | 3066·3 | 2176·3 | 1376·4 | 2836·5 | | | CT |
| | 3457·6 | 2862·5 | 2825·1 | 3868·7 | 2479·9 | 1986·6 | 1570·4 | 2684·6 | | | M |
| | 468·1 | 315·8 | 403·8 | 557·3 | 615·1 | 172·4 | 181·5 | 136·1 | | | s.d. |
| | 27·0 | 16·8 | 16·2 | 16·9 | 10·3 | 1·8 | 0 | 13·7 | | | CYT |
| 4 | 7893·4 | 7964·5 | 7367·3 | 7864·7 | 6947·3 | 2037·4 | 1539·4 | 2538·6 | | | CT |
| | 7268·3 | 8063·4 | 7659·4 | 7956·4 | 7047·9 | 1903·4 | 1477·9 | 2675·3 | | | CT |
| | 7049·1 | 7865·4 | 7866·3 | 7863·5 | 6993·8 | 1864·3 | 1626·9 | 2330·5 | | | CT |
| | 7403·6 | 7964·4 | 7631·0 | 7894·9 | 6996·3 | 1935·0 | 1548·1 | 2514·8 | | | M |
| | 438·1 | 99·0 | 250·7 | 53·3 | 50·3 | 90·8 | 74·9 | 173·6 | | | s.d. |
| | 94·6 | 104·2 | 98·5 | 102·9 | 87·6 | 1·0 | 0 | 10·8 | | | CYT |
| 8 | 7793·4 | 7986·3 | 7493·6 | 7649·6 | 6849·7 | 1830·6 | 1837·6 | 2783·7 | | | CT |
| | 7849·8 | 8040·1 | 7683·6 | 7756·3 | 7003·9 | 1824·3 | 1584·6 | 2203·9 | | | CT |
| | 7648·3 | 7865·4 | 7796·5 | 7638·9 | 7536·4 | 1786·0 | 1639·8 | 2478·3 | | | CT |
| | 7763·8 | 7963·9 | 7657·9 | 7681·6 | 7130·0 | 1813·6 | 1687·3 | 2488·6 | | | M |
| | 103·9 | 89·5 | 153·1 | 64·9 | 360·3 | 24·1 | 133·0 | 290·0 | | | s.d. |
| | 100·7 | 104·2 | 98·9 | 99·3 | 89·9 | 0 | 0 | 10·4 | | | CYT |

Spont, Spontaneous lysis; max, maximal lysis; CT, ct/min; M, mean; CYT, calculated specific cytotoxicity.

9777Sk) pretreated with TNF-$\alpha$ (1 ng/ml) for 2 h in the presence of preactivated neutrophils (control).

In addition, a cytolytic effect of effector cells alone (without antibodies) was investigated (control).

## RESULTS

Five selected out of 50 WG sera were monospecifically positive for anti-PR-3 antibodies as determined by IFT on human neutrophils (C-ANCA$^+$; titres 1 : 80–1 : 640), by ELISAs with alpha fraction and 'acid' extract of human neutrophils and total extract of cytokine-treated HEC, by Western blot (reaction at 29 kD) and other routine methods [11] (data not shown). F(ab')$_2$ fragments of the affinity-purified antibodies reacted positively in an ELISA and in Western blot (data published earlier) [4,22]. Antibody reactivity could be blocked by incubation with purified PR-3 antigen (affinity-purified as well as Kao preparation) as measured by ELISA and determined by Western blot [4]. Antibody reactivity could not be inhibited by preincubation with extracts of HEp2 cells. In addition, four sera of patients with Sjögren's syndrome (SS) and one serum of a patient with Sharp syndrome were found to be positive for anti-Ro (SS-A), anti-La (SS-B) and anti-RNP antibodies. Antibodies of these sera were purified and served as controls.

Antibody preparations diluted to the highest concentrations used in the experiments were free of endotoxin as determined by a limulus amoebocyte lysate assay.

Considering the possible relevance of an anti-PR-3 antibody-mediated cytotoxicity we investigated the effect of coincubation with neutrophils. Figure 1a–c shows the results of the cytotoxicity assay after addition of IFN-$\gamma$-treated neutrophils as effector cells. Incubation of HEC, pretreated with TNF-$\alpha$ for 2 h with affinity-purified anti-PR-3 antibodies and IFN-$\gamma$-primed neutrophils, actuated a maximal cytotoxic effect after 4 h (Fig. 1a,b). This effect is dependent on the concentration of anti-PR-3-IgG and is endothelial cell-specific (Fig. 1f,g). There is no relation of disease activity to cytotoxic potency of anti-PR-3 preparations. To demonstrate specificity of antibody effect, several control experiments with different antigen preparations were performed (Fig. 1c,d). Anti-PR-3 antibodies preincubated with alpha fraction of granulocytes or Kao preparation of PR-3 as well as neutrophils without anti-PR-3 antibodies caused no cytotoxic effect. Other antibody specificities (controls) such as dsDNA, SS-A/Ro and SS-B/La, were not able to kill HEC pretreated for 2 h with TNF-$\alpha$ (Fig. 1e). The observed cytotoxic reaction was not complement-dependent (Fig. 1b). An important prerequisite for a cytotoxic effect of anti-PR-3 antibodies was the TNF-$\alpha$-induced membrane expression of PR-3 (data not shown) [4].

Our results show that in contrast to other antibody specificities (dsDNA, SS-A/Ro, SS-B/La, ELAM-1), cytotoxic effects were only obtained by anti-PR-3 antibodies. Cytotoxic effects only occurred after membrane expression of PR-3 on HEC induced by TNF-$\alpha$ [4] and in the presence

**Table 3.** Data of Fig. 1f and g

| Time (h) | WG1 2·0 mg/ml | WG1 1·0 mg/ml | WG1 0·5 mg/ml | WG1 0·1 mg/ml | WG2 2·0 mg/ml | WG2 1·0 mg/ml | WG2 0·5 mg/ml | WG2 0·1 mg/ml | Fibrobl. WG2 2·0 mg/ml | Spont. | Max. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2348·4 | 2278·2 | 2154·3 | 2039·2 | 2465·8 | 2365·1 | 2102·7 | 2036·2 | 1835·4 | 1633·3 | 7321·8 | CT |
| | 2149·7 | 2167·9 | 2200·7 | 2187·1 | 2410·2 | 2210·7 | 2197·1 | 2008·3 | 1764·3 | 1748·5 | 7203·5 | CT |
| | 2284·3 | 2143·8 | 2143·8 | 2243·4 | 2186·5 | 2267·8 | 2101·7 | 2187·0 | 1745·6 | 1793·8 | 7211·4 | CT |
| | 2260·8 | 2196·6 | 2166·3 | 2156·6 | 2354·2 | 2281·2 | 2133·8 | 2077·2 | 1781·8 | 1725·2 | 7245·6 | M |
| | 82·8 | 58·5 | 24·7 | 86·1 | 120·7 | 63·7 | 44·7 | 78·5 | 38·7 | 67·6 | 54·0 | s.d. |
| | 9·7 | 8·5 | 8·6 | 7·8 | 11·4 | 10·0 | 7·4 | 6·4 | 1·0 | | | CYT |
| 2 | 2488·5 | 2365·3 | 2194·5 | 2068·1 | 2531·2 | 2200·9 | 2230·1 | 2103·8 | 2001·5 | | | CT |
| | 2567·9 | 2283·1 | 2038·1 | 2102·1 | 2466·9 | 2375·1 | 2143·8 | 2075·9 | 2156·3 | | | CT |
| | 2599·8 | 2199·5 | 2177·9 | 2245·3 | 2369·3 | 2189·7 | 2099·5 | 2089·5 | 2198·2 | | | CT |
| | 2552·1 | 2282·6 | 2136·8 | 2138·5 | 2455·8 | 2255·2 | 2157·8 | 2089·7 | 2118·7 | | | M |
| | 46·8 | 67·7 | 70·1 | 76·8 | 66·6 | 84·9 | 54·2 | 11·4 | 84·6 | | | s.d. |
| | 14·9 | 10·0 | 7·4 | 7·5 | 13·2 | 9·6 | 7·8 | 6·5 | 7·1 | | | CYT |
| 4 | 6910·2 | 5654·9 | 5043·2 | 4367·5 | 7436·1 | 5910·2 | 4669·2 | 4146·7 | 2210·2 | | | CT |
| | 7385·3 | 5672·8 | 4975·1 | 4288·9 | 7436·7 | 6129·8 | 4753·2 | 4288·5 | 2253·7 | | | CT |
| | 7238·1 | 5531·7 | 5079·1 | 4326·3 | 7533·9 | 5853·2 | 4690·2 | 3993·2 | 2273·2 | | | CT |
| | 7177·9 | 5619·8 | 5032·5 | 4327·6 | 7468·9 | 5964·4 | 4704·2 | 4142·8 | 2247·7 | | | M |
| | 198·6 | 62·7 | 43·1 | 32·1 | 45·9 | 119·2 | 35·7 | 120·6 | 23·6 | | | s.d. |
| | 98·8 | 70·5 | 59·9 | 47·1 | 104·0 | 76·8 | 53·9 | 43·8 | 9·5 | | | CYT |
| 8 | 7201·3 | 6329·1 | 5238·4 | 4307·2 | 7530·3 | 5993·1 | 5133·0 | 4153·6 | 2345·3 | | | CT |
| | 7149·2 | 6244·3 | 5291·2 | 4299·7 | 7478·6 | 6051·8 | 5101·2 | 4177·0 | 2351·2 | | | CT |
| | 7320·7 | 6386·1 | 5102·4 | 4310·2 | 7343·9 | 5912·5 | 5036·2 | 4093·8 | 2240·3 | | | CT |
| | 7223·7 | 6319·1 | 5210·7 | 4305·7 | 7450·9 | 5975·8 | 5090·1 | 4141·5 | 2315·3 | | | M |
| | 71·8 | 58·3 | 79·5 | 4·4 | 78·6 | 46·3 | 40·3 | 35·0 | 50·9 | | | s.d. |
| | 99·6 | 83·2 | 63·1 | 46·7 | 103·7 | 76·9 | 60·9 | 43·7 | 10·6 | | | CYT |

Spont, Spontaneous lysis; max, maximal lysis; CT, ct/min; M, mean; CYT, calculated specific cytotoxicity.

of IFN-γ-primed neutrophils. Cytotoxic effects were independent of complement.

## DISCUSSION

Endothelium has often been considered a possible target of an immune-mediated aggression during vasculitic processes, due to its functional characteristics and because it is constantly in contact with circulating humoral and cellular immune effectors [23].

Antibodies against endothelial cells (AECA) have been described in various connective tissue diseases [24,25]. AECA in sera of a small number of patients with systemic vasculitis have previously been reported. The pathogenic significance and the different antigen specificities of these antibodies have not yet been clarified, but the interaction of circulating autoantibodies with the endothelium may be an important mechanism in the pathogenesis of systemic vasculitis. Leung *et al.* were able to demonstrate in Kawasaki syndrome IgG and IgM-AECA that killed cytokine-treated endothelial cells from umbilical cord veins, but had no effect on untreated resting cells [26]. In this disease, excess monocyte cytokine release is followed by endothelial cell activation and lysis by circulating antibodies. Leung *et al.* were also able to demonstrate a complement-dependent cytotoxic effect of IgM antibodies against HEC in sera of patients with Kawasaki syndrome [27]. A remarkable feature was that HEC had to be pretreated with IFN-γ. The authors concluded that these AECA in Kawasaki sera could be directed against cytokine-induced surface structures, and assumed that this could be an important mechanism in the pathogenesis of vasculitides. Cotran *et al.* assumed that endothelial neoantigens, known to be expressed in response to cytokines, may be additionally required [28].

Abbott *et al.* found by indirect immunofluorescence that both a MoAb to neutrophil cytoplasmic antigens and WG sera bound to human cultured glomerular epithelial and endothelial cells and also to endothelial cells obtained from umbilical cord vein. The authors concluded that the antigen recognized by the ANCA-positive sera is also expressed on surface vascular structures, suggesting a direct pathogenic role for these antibodies [29]. Recently we detected PR-3 in HEC, and showed that PR-3 is translocated into the HEC membrane under the influence of cytokines, and is accessible to C-ANCA [4]. Anti-PR-3 antibodies therefore represent a subgroup of AECA.

At present the exact mechanism of a direct pathogenic effect of anti-PR-3 antibodies remains an enigma, and there is no definitive proof as yet for a causative role of ANCA in ANCA-related diseases. There are some data, however, generated from *in vitro* experiments, pointing to a possible pathophysiological role of ANCA in ANCA-related vasculitides (for review see [5]). A favoured hypothesis discusses a putative direct cytotoxic effect of ANCA towards HEC. Holt *et al.* demonstrated ADCC towards HEC with sera of patients suffering from systemic

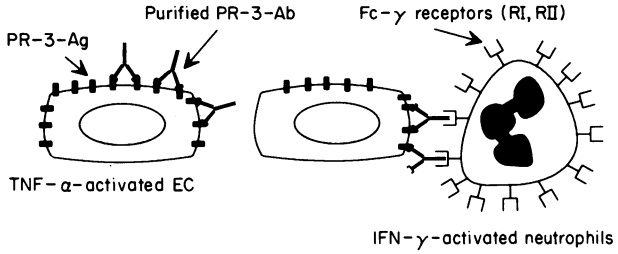


**Fig. 2.** Mechanism of anti-proteinase 3 (PR-3) antibody-dependent cellular cytotoxicity. EC, Endothelial cells.

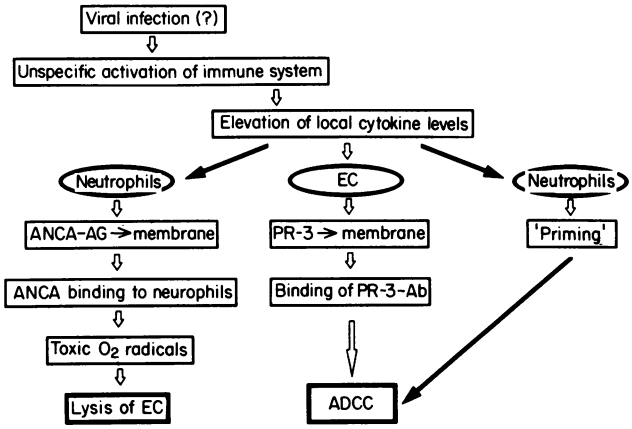


**Fig. 3.** Model of pathogenic effects of anti-proteinase 3 (PR-3) antibodies in Wegener's granulomatosis and ANCA-related vasculitides. EC, Endothelial cells.

sclerosis coincubated with peripheral blood monocytes [30]. These findings confirmed data of Penning *et al.*, who assumed that AECA could represent an important factor in ADCC of vascular endothelial cells [31,32]. Brasile *et al.* described AECA that were cytotoxic in the presence of complement to monocytes and HEC [33]. AECA found in WG sera seem not to have any complement-mediated effects on endothelial cells [23,29,34]. These results have been confirmed by Savage *et al.* Some of their serum samples demonstrated antibody-dependent, mononuclear cell-mediated cytotoxicity [35]. Recently Savage *et al.* reported the ability of ANCA (i.e. anti-PR-3 and anti-MPO antibodies) to activate neutrophils and mediate cytotoxicity against vascular endothelium. Preactivation of endothelial cells with TNF enhanced this effect. Binding of ANCA to target antigens expressed in the membrane of endothelial cells was not investigated. The authors discuss a better activation of neutrophils or an enhanced vulnerability of preactivated endothelial cells against injurious substances as possible mechanisms of the cytotoxic effect [36].

In our study we were able to show a cytotoxic effect of anti-PR-3 antibodies towards HEC. HEC pretreated with TNF-$\alpha$ were killed *in vitro* in the presence of anti-PR-3 antibodies and IFN-$\gamma$-activated neutrophils. This cytotoxic effect is not dependent on complement and equivalent to ADCC. An important prerequisite of ADCC is binding of Fc receptors on an effector cell (i.e. IFN-$\gamma$-primed neutrophil) to an antibody-coated target cell (i.e. HEC after binding to anti-PR-3 antibodies) (Fig. 2). Until now few models of ANCA-mediated vasculitis have existed. The ANCA–cytokine sequence theory proposes a translocation of polymorphonuclear neutrophil (PMN) proteases *in vivo* to the surface of cytokine-activated PMN, thus making them accessible to ANCA [37]. Cytokines also lead to the expression of adhesion molecules on HEC, allowing a close contact of PMN with HEC [38]. ANCA bind to and further activate PMN, which results in a respiratory burst and degranulation, thus inducing vascular injury [39]. Similarly, in another model HEC membranes become direct targets for the autoimmune reaction: activated PMN first release proteases, which then bind to HEC membranes [36].

Data of the present study show endothelial cells again as active protagonists in pathogenesis. Detection of PR-3 in HEC, their membrane expression [4] and the described direct effects of anti-PR-3 antibodies on HEC point to a possible mechanism of pathogenic events as depicted in Fig. 3. An important aspect are the complex interactions between cytokines, antibodies, neutrophils and HEC. Locally elevated levels of cytokines make PR-3 accessible on the surface of neutrophils and HEC for the respective antibodies. Anti-PR-3 antibodies trigger endothelial cytotoxicity via priming of neutrophils with subsequent production of toxic $O_2$ radicals as well as via ADCC. Moreover, since anti-PR-3 antibodies are capable of exerting cytokine-like effects on HEC (i.e. induction of E-selectin expression) [22], a kind of 'vicious circle' may occur.

This is to the best of our knowledge the first report on direct cytotoxic effects of anti-PR-3 antibodies on HEC. Our data give a hint of an anti-PR-3 antibody-mediated mechanism of endothelial injury via ADCC in WG and other ANCA-related vasculitides.

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