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Anti-granulocyte antibodies in immune neutropenia and autoimmune disorders

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Anti-granulocyte antibodies may have an important role in the pathophysiology of several immunological diseases. These include (1) drug-induced granulocytopenia, (2) alloimmune neutropenia, (3) primary autoimmune neutropenia, (4) neutropenias secondary to autoimmune disorders and (5) anti-granulocyte antibodies without neutropenia [8].

The pathological role of anti-granulocyte antibodies in immune mechanisms of neutropenia is proven in Felty's syndrome [11]. Similar results in patients with other autoimmune diseases are controversial [8].

In the recent study we investigated the occurrence, the effect and specificity of anti-granulocyte antibodies in patients with systemic lupus erythematosus (SLE) compared to those with progressive systemic sclerosis (PSS).

Patients and Methods

Patients

144 patients with SLE (131 female, 13 male, mean age: 48.3 ± 14.6 yrs) who fulfilled the ARA criteria (13) were examined in a 4 years' follow up study. 300 examination were performed for anti-granulocyte antibodies (AGAs). In 25% of the cases, patients were studied in an anctive phase of the disease. During the examinations all of patients received low doses of prednisone (5–30 mg/die). The percents of several organ involvements and laboratory findings corresponded to the data of previous reports.

38 female patients with PSS (mean age: 47.8 ± 11.7 yrs) were also investigated. All of our patients fulfilled the preliminary criteria for scleroderma (12). They were treated with nifedipine (30–50 mg/die), D-penicillamine and low doses of prednisone.

46 healthy controls (39 female, 7 male, mean age: 45.6 ± 13.6 yrs) were also investigated and analysed synchronously with the probands.

Cell separations

Granulocytes and mononuclear cells were isolated from heparinised blood of healthy donors [2]. The separated cells showed a viability of 97%.

Platelets were separated from peripheral blood anticoagulated with EDTA.

Granulocytotoxicity test

A microgranulocytotoxicity test was used by applying the method of Hasegawa et al. [5].

Enzyme linked immunosorbent assay (ELISA)

For determination of anti-granulocyte, anti-monocyte and anti-platelet autoanti-

bodies, the method of Voller et al. [16] was used, as modified by Doughty et al. [4], described previously in details by us [10].

Anti-DNA antibodies were measured by modification of Weetman et al. [17].

A positive antibody result was taken as one 2SD or more above the control mean at 1: 100 serum-dilutions.

Chemotaxis assay

Granulocyte chemotaxis was measured by the two cell leading front assay in modified Boyden chambers [7], for detecting the effect of AGAs positive sera on chemotaxis of normal granulocytes. Healthy granulocytes were pretreated with heat-inactivated patients' or control sera diluted in 1: 5 [10].

Yeast phagocytosis and opsonised yeast phagocytosis

A monolayer technique was used with pretreated granulocytes adherent to a glass slide [10]. Baker's yeast suspension or yeast particles opsonised by human AB serum were applied to the tests. Phagocytic index (PI) was calculated.

Leukocyte adherence inhibition (LAI)

Capillary tube leukocyte adherence inhibition was performed [15]. Healthy granulocytes were pretreated with sera as mentioned above. Cell counting was done by Picoscale (PS-4, Medicor, Hungary).

Assay for oxygen consumption

Oxygen consumption was measured polarographically. Corning pO_2 electrode, a modified Clark-type oxygen electrode (Radelkis, Hungary) is attached to a basic analyser (Typ OP 210/3, Radelkis, Hungary), which registrate actual partial O_2 tension (Hgmm) by using a polarograph (Typ OH-814/1 Radelkis, Hungary).

The O_2 electrode is in direct contact with a polystyrene dish containing the granulocyte suspension (2 ml of 10^7/ml), and it markes continously the changes of the O_2 tension of the fluid phase. The whole system is opened, therefore the initial O_2 pressure of the fluid phase corresponds to the atmospheric O_2 tension. The fast decrease of the initial O_2 pressure (158 Hgmm) correlates to the O_2 consumption of phorbol-mystrate-acetate (PMA) (5 mg of 10^{-7} nmol/l) activated, pretreated normal granulocytes. The consumption was followed and registrated for 10 minutes, and O_2 consumption rate was calculated in every 2 minutes:

$$O_2 \text{ cons. rate: } \frac{O_2 \text{ cons. of pretreated cells}}{O_2 \text{ cons. of control cells}}$$

Inhibitory indirect immunofluorescence test

This test was used for detecting the inhibitory effect of AGAs on binding of several monoclonal antibodies (mAbs). The panel of mAbs is as follows:

1. 44 (CD 11 = LFA-α chain)
2. VIMD5 (CD 15A = X hapten)
3. GF. 26. 7. 3 (CD 15B)
4. 8—27-F6 (CD 15C)
5. FcRgranI (CD 16 = FcR_{low})
6. MHM23 (CD 18 = LFA-β chain)
7. Ell (CD 35 = CR1)
8. 48 (LCA)

The effect of 12 AGA positive SLE sera and 7 AGA positive PSS sera were tested. Normal granulocytes were pretreated with these sera, then preincubated with the mAbs diluted in 1: 200. Fluorescein-isothiocyanate (FITC) conjugated, affinity purified $F(ab)_2$ fragment of goat anti-mouse Ig in dilution 1: 20 (Cappel Cooper Diagnostic, Cochranville, Pennsylvania) was added. The results were evaluated by Zeiss Fluorescence microscope, and were given in % of stained cells. Positive inhibitory effect was taken above 20%.

Western immunoblotting technique for detection of specific anti-granulocyte antibodies

Soluble plasmamembrane preparations isolated from unactivated (I) and PMA activated (II) human normal granulocytes were used as antigens, produced by Borregaard et al. [1].

6 AGA positive SLE sera (diluted in 1: 10) were tested in this method. Immunoreplica analysis was performed [6, 14]. Peroxydase-conjugated goat anti-human IgGAM (Human Institute, Hungary) diluted in 1: 200 was applied to develop the positive reaction. Apparent mol wts were estimated by references to molecular markers [ie. 14, 20, 43, 67 and 94 kD].

Statistical analysis

The same variables of different groups were tested by the non-parametric Mann-Whitney-Wilcoxon test. The differences between the groups were considered statistically significant when $p < 0.05$.

Results and Discussion

During a long term follow-up study the frequency of AGAs proved to be 32% in patients with SLE. Out of these, 67% showed cytotoxic effect. Considering the clinical observations and laboratory parameters, there was found a close connection between the occurrence of AGAs and the clinical activity of the disease ($p < 0.01$). In cases of renal involvement the presence of AGAs is more frequent ($p < 0.05$). However these antibodies do not cause granulocytopenia. A highly significant common occurrence ($p < 0.001$) of LE phenomenon and AGAs could be detected. This may indicate a site of the impotance of AGAs. The presence of AGAs showed also relationship to the anti-DNA, anti-platelet and anti-monocyte antibodies ($p < 0.01$).

Considering the effect of AGA positive sera on healthy granulocytes, no changes in adherence inhibition could be detected. Inhibition of yeast phagocytosis and chemotaxis was detected, but irrespective of the presence of AGAs. SLE sera containing AGAs gave markedly reduced Fc-receptor dependent opsonised yeast phagocytosis ($p < 0.001$). The kinetics of the oxygen consumption of normal granulocytes in the presence of AGAs is similar to the effect of SLE sera without AGAs, but the antibodies delayed starting of the O_2 consumption and reaching the plateau within 10 minutes.

The AGAs were found in 21% of 38 patients with PSS. 85% of the positive sera showed granulocytotoxic activity [9, 10]. No connection with granulocytopenia was found. Sicca syndrome and myositis were not found in patients with AGAs. No correlation to other clinical symptoms nor to other laboratory findings were detected.

Spontaneous adherence of healthy granulocytes was not influenced by PSS sera. Chemotaxis was inhibited independently of the presence of AGAs. Yeast phagocytosis and opsonised yeast phagocytosis were definitely reduced by sera containing AGAs ($p < 0.01$). The O_2 consumption of normal granulocytes in the presence of AGAs were significantly reduced ($p < 0.01$) compared to those without AGAs. These results indicate that the effects of AGAs is very complex, they probably interfer not only with phagocytosis, but also may influence more general functions, ie. oxydative metabolism.

We also investigated the antigen/receptor specificity of AGAs in the sera of SLE and PSS sera. First the inte-

raction of 12 SLE and 7 PSS sera, all positive for AGAs, were defined by inhibiting the binding of different mAbs in immunofluorescence test. More than 50% of the studied SLE and PSS sera revealed binding to CD 15 (X hapten) and/or CD 16 (FcR_{low}) antigens. This observation was completed by western immunoblotting technique for visualization of granulocyte membrane proteins specific for AGAs. The binding of AGAs to bands with mol wts about 50–60 kD and near to 30 kD could be demonstrated from 4 out of 6 AGA positive SLE sera. The intensity of the components were varied according to the tested sera. These latter results correspond to the finding of inhibitory immunofluorescence test, since CD 16 is a 50–60 kD protein, and CD 15C possess also proteins of 28 and 32 kD. The loss of 50–60 kD band on the PMA activated granulocyte membrane preparation is still unclear, it may occur as receptor internalisation after PMA activation [3].

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