Human neutrophil activating peptide/interleukin 8 acts as an autoantigen in rheumatoid arthritis

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Abstract

Human neutrophil activating peptide/interleukin 8 (NAP-1/IL-8) has been shown to activate neutrophils to degranulate in vitro and to be a potent chemotactic agonist for neutrophils and lymphocytes in vitro and in vivo. It may therefore be a mediator of inflammatory conditions such as rheumatoid arthritis (RA).

Levels of NAP-1/IL-8 were low or undetectable in serum samples from 53 patients with RA. Circulating levels of antibodies to NAP-1/IL-8 showed a strong correlation with the level of quantified C reactive protein and with the number of arthritic joints. These autoantibodies, in a similar manner to quantified C reactive protein, correlated with disease activity and are associated with a lack of clinical improvement when the patient is treated with systemic steroids.

This observation indicates an important role for interleukin 8 and its autoantibodies in the inflammatory processes of RA, and may provide a clinically useful marker for the diagnosis of disease severity.

Human neutrophil activating peptide/interleukin 8 (NAP-1/IL-8) is a 72 amino acid peptide which has been shown to activate neutrophils to degranulate and show respiratory burst in vitro,^{1,2} and to be chemotactic for neutrophils and lymphocytes in vitro and in vivo.^{3–6}

Neutrophils are a major source of enzymes, including those which degrade cartilage,⁷ and the accumulation of these cells in tissues is often observed in a variety of pathological conditions. These observations led us to examine circulating levels of the cytokine and antibodies directed against it in patients with rheumatoid arthritis (RA), and the development of a solid phase double ligand enzyme linked immunosorbent assay,⁸ (ELISA) has enabled quantitative determinations of these parameters in serum samples.

Serum samples from 53 patients with definite or classical RA as defined by the American Rheumatism Association criteria⁹ were tested for NAP-1/IL-8 and antibodies to NAP-1/IL-8. The clinical examinations were performed by a single observer.

The assay results were correlated with levels of quantified C reactive protein and with the number of arthritic joints, characterised by inflammation, pain, stiffness, swelling, and impairment. The levels of C reactive protein provide an objective index of disease activity in patients with RA, as high levels are associated

with progressive disease, whereas the induction of clinical remission and control of the underlying disease process is associated with prompt normalisation of the level of C reactive protein.

Methods

Quantified C reactive protein was determined by the standard nephelometric method of Whicher *et al.*¹⁰

Goat polyclonal and mouse monoclonal antibodies to NAP-1/IL-8 were purified by immunosorption on a recombinant NAP-1/IL-8-Sepharose column. The two types of antibody bind to NAP-1/IL-8 in Western blots, showing a strong band at 8.5 kilodaltons, and both inhibit NAP-1/IL-8 induced elastase release from freshly isolated human neutrophils (data not given).

NAP-1/IL-8 was determined by a solid phase double ligand ELISA with a quantitative evaluation range of 0.2–25 ng/ml.⁸ Briefly, wells of microtitre plates were coated with immunosorbent purified polyclonal goat or mouse monoclonal antibodies to NAP-1/IL-8 in 0.1 M sodium carbonate/sodium hydrogen carbonate buffer (pH 9.6) for 16 hours at 4°C. After four washes with phosphate buffered saline (pH 7.5), the serum samples were added to the wells and incubated for two hours at 37°C. After four washes, goat anti-NAP-1 alkaline phosphatase conjugate was added and the plates were incubated for a further two hours at 37°C. After the addition of *p*-nitrophenyl phosphate and incubation the reaction was terminated by the addition of 2 M sodium hydroxide solution and the absorption measured at 405 nm. The bound NAP-1 was determined by comparison with a standard.

To define the specificity of this ELISA we tested several peptides with high homology to NAP-1/IL-8 for cross reactivity. At concentrations up to 1000 ng/ml, connective tissue activating peptide 3, platelet factor 4, and neutrophil activating peptide 2 show no cross reactivity with NAP-1/IL-8 (data not given).

Circulating antibodies to NAP-1/IL-8 were determined by a similar method, in which the wells of microtitre plates were coated with NAP-1/IL-8 at a concentration of 5 µg/ml and stored overnight at 4°C. The wells were then washed four times with 0.05% Tween 20, and 100 µl amounts of serum samples (diluted 1:10) were added and incubated at 37°C for two hours. After four washings, 50 µl of goat antihuman IgG alkaline phosphatase conjugate (Bio-Rad) was added at a concentration of 5 µg/ml and the plates again incubated for two

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hours at 37°C. The enzymatic reaction was initiated by the addition of *p*-nitrophenyl phosphate as before, and the absorption was determined at 405 nm after termination of the reaction with 2 M sodium hydroxide solution. As no standard human IgG antibody to IL-8 is available, the absolute concentrations could not be defined. Antibody levels are therefore expressed as density units from the ELISA to give values of relative concentration. The antibodies in serum samples, which recognise the purified recombinant NAP-1/IL-8 bound to the plate, also recognise the bound NAP-1/IL-8 on the immunosorbent column, and can be quantitatively removed from the serum sample on this column.

Regression analysis of the results was performed with the RS1 statistics programme from BBN Software Products.

Lipopolysaccharides were determined by an automated system.¹¹

Results

Table 1 gives the age, sex, disease stage, and treatment of the studied patients. Of 53 serum

Table 1 Age, sex, disease stage; and treatment of patients included in this study. Figures given for disease stage and treatment are numbers of patients

Mean age in years (range)	54.2 (27–82)
Sex (women/men)	33/20
Mean duration of disease in years (range)	6.5 (0.6–24)
Disease status:	
Steinbrocker I	21
Steinbrocker II	25
Steinbrocker III	6
Steinbrocker IV	1
Rheumatoid factor positive/negative	27/26
Treatment:	
Antimalarial drugs	15
Gold salts	16
D-Penicillamine	5
Steroid/ACTH*	11
Immunosuppressive drugs	17

*ACTH=adrenocorticotrophic hormone.

samples, 14 (26%) contained NAP-1/IL-8 at levels of 0.27–4.6 ng/ml and no significant difference in the levels was seen in samples taken 8 and 20 days later (data not given). There was no correlation between these low NAP-1/IL-8 concentrations and disease severity. In all patients, irrespective of the NAP-1/IL-8 concentration, a low lipopolysaccharide concentration of approximately 10 pg/ml was found.

No NAP-1/IL-8 was found in serum samples from nine healthy controls, and these controls defined the background titre range of 0.089–0.307 absorption units for antibodies to NAP-1/IL-8. These patients had low circulating levels of quantified C reactive protein between 8 and 13 mg/l.

Similar background levels of antibodies to NAP-1 (0.106–0.296 absorption units) were detected in nine patients with RA who were either in complete remission or showing a significant improvement as a result of treatment. In patients with active disease, however, higher levels of antibody were detected. Figure 1 shows the highly significant relationship between levels of antibody to NAP-1 and quantified C reactive protein (fig. 1A) and the number of arthritic joints in all patients (fig. 1B). Table 2 gives the statistical correlations for these data. No significant correlation was seen between antibody levels and the duration of disease, and, similarly, there was no significant difference in titres of antibody to IL-8 between rheumatoid factor positive and negative patients.

Figure 2 shows the titres for IgG antibody to NAP-1/IL-8 in serum samples from the same patients divided into treatment groups. There is a tendency towards higher antibody levels in the patients treated with immunosuppressive drugs and D-penicillamine. These drugs are generally regarded as second line treatment, used for patients where the disease appears to be resistant to initial treatment with gold or

Figure 1 Relationship between levels of IgG antibodies to NAP-1/IL-8 in serum samples and (A) quantified C reactive protein or (B) number of arthritic joints. Points represent individual patients. Antibody levels are given as units of absorption at 405 nm (OD₄₀₅) from the ELISA (see under Methods). Dotted lines represent 95% confidence limits.

Table 2 Statistical correlations between IgG antibodies to NAP-1/IL-8, number of arthritic joints and quantified C reactive protein. $p < 0.001$

	Quantified C reactive protein	No of arthritic joints
Arthritic joints	0.859965	
Antibodies to NAP-1/IL-8	0.788961	0.747471

Figure 2 Levels of IgG antibodies to NAP-1/IL-8 in patients with RA divided into treatment groups. (c) Normal control subjects (n=9). (g) Patients receiving 6 mg auranofin daily by mouth (n=16). (i) Patients receiving immunosuppressive treatment (n=5); two received 25 mg methotrexate weekly, and three received 100 mg azathioprine daily. (i/s) Patients receiving immunosuppressive treatment plus steroids (n=11); three patients received 1 mg ACTH (tetracosactid) weekly plus 25 mg methotrexate weekly, eight received 8–20 methylprednisolone daily, of whom four also received 100 mg azathioprine daily, and four received 25 mg methotrexate weekly. (p) Patients receiving penicillamine, 150–500 mg daily (n=5). (r) Patients receiving treatment with Resochin; 250 mg chloroquine daily (n=15). Points represent individual patients. Antibody levels are given as units of absorption at 405 nm (OD₄₀₅) from the ELISA (see under Methods).

chloroquine. The most potent treatment is the combination of immunosuppressive drugs and steroids, and in this group a very marked increase in antibody concentrations was seen. The patients in this group also showed a poor response to treatment.

Discussion

In the rheumatoid serum samples tested there was a clear correlation between the serological and clinical parameters indicative of disease and the presence of autoantibodies against IL-8. This observation, together with the low or undetectable IL-8 titres in patients with raised levels of quantified C reactive protein and antibodies suggests a possible interaction between IL-8 and its autoantibody, and could indicate a major role for IL-8 and antibodies to IL-8 in this disease.

Interleukin 1 and tumour necrosis factor are both potent stimulators of IL-8 production from a variety of cell types,^{7 12 13} possibly providing another fundamental link in the chain of reactions leading to inflammation. In RA generated by an immune response to an as yet unidentified antigen, the involvement of monocytes and lymphocytes and their secretion of inflammatory and chemotactic factors has been well documented.¹⁴

Reports of autoantibodies to cytokines are relatively rare, although titres of antibody to

interleukin 1 have been reported in a small proportion (17%) of patients with RA,¹⁵ and Fomsgaard *et al* have described antibodies directed against tumour necrosis factor in patients with various rheumatic diseases.¹⁶ These authors did not, however, show a correlation between antibody levels and disease severity, as is seen here.

The specific function of such circulating antibodies to cytokines is unclear. It has been speculated that they could represent a mechanism of cytokine regulation, acting as physiological carriers of the cytokine, or could fulfil an inhibitory role, neutralising unwanted activities.¹⁷ It is possible that such a regulatory influence could be applied to NAP-1/IL-8. This cytokine has the potential to play a major role in RA, as it appears to be produced at high concentrations in the affected joints of patients with RA. We have detected NAP-1/IL-8 in the synovial fluid of patients with RA at levels which correlate extremely well with disease severity, determined by either the concentration of quantified C reactive protein in serum samples, or the total cell count in the synovial fluid.¹⁸

NAP-1/IL-8 could be either a stimulator of the disease processes of RA, or a direct result of these processes. Its release from the affected joint would result in high levels of IL-8 in the circulation, followed by an increase in autoantibodies. This would explain why levels of antibody to NAP-1/IL-8 correlate with disease severity. The higher the titre, the stronger the treatment needed to induce remission or even to stabilise the disease, for these high titres are associated with a lack of clinical improvement under treatment with systemic steroids, and are indicative of a need for immunosuppressive treatment, or even combination treatment with immunosuppressive drugs and steroids.

These observations indicate an important role for IL-8 and its autoantibodies in the inflammatory processes of RA, and may provide a clinically useful marker for the diagnosis of disease severity.

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