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Characterization of naturally occurring autoantibodies against tumour necrosis factor-alpha (TNF-α): *in vitro* function and precise epitope mapping by phage epitope library

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SUMMARY

Naturally occurring autoantibodies against cytokines exist in the sera of patients with autoimmune diseases as well as in the sera of normal individuals. We report here that affinity-purified autoantibodies against human TNF-α from one rheumatoid arthritis (RA) patient inhibited the cytotoxic effect of TNF-α on the mouse fibrosarcoma cell line WEHI 164, by 50%. In an attempt to predict the autoantibodies' recognition site on TNF-α protein we screened a random nanopeptide phage library with the affinity-purified TNF-α autoantibodies. Among 63 random selected clones, 46 clones carried the sequence ASSLLASSP, NSSPYLNTK or PQSPGSSFP. Frequency analysis of the relative occurrence of the 20 amino acids in the nanopeptides displayed by 50 random bacteriophages picked before selection and 63 after selection to bind to TNF-α autoantibodies indicated that proline ($P < 0.0003$) and serine ($P < 0.04$) are involved in the binding of the autoantibodies to the phages. Furthermore, we demonstrated that three synthetic peptides (ASSLLASSP, NSSPYLNTK and PPLKPVIDE) displayed by the selected phages reduced the binding of the autoantibodies to TNF-α protein by 50%. Interestingly, the sera of mice (BALB/c) immunized with phages displaying ASSLLASSP and NSSPYLNTK peptide showed an anti-TNF-α response as detected by ELISA. This response was not found in mice immunized with the wild type phage. Thus, the recombinant phages selected from the phage libraries could be used as carrier for immunization, and therefore as a tool for vaccine development. This work sets the stage for experiments designed to isolate ligands for protective antibodies.

Keywords autoantibodies mapping library tumour necrosis factor-alpha

INTRODUCTION

Cytokines are small proteins produced by various types of cells, and they mediate growth, differentiation and activation of the same (autocrine) or other (paracrine) cells. These substances have important roles in normal physiology as well as in host defence mechanisms of the immune and inflammatory system [1]. Rheumatoid arthritis (RA) is an inflammation of the joint in which the patient exhibits an inappropriate immune response which results in progressive damage [2]. Substantial amounts of IL-1, TNF-α and IL-6 are found in the joints of RA patients [2–4]. TNF-α has shown to be one of the major mediators in the regulation of the inflammatory response. Indeed, mice studies provided further insight into the importance of TNF-α in arthritis [5,6]. Keffler *et al.* described a mouse transgenic for human TNF-α gene, which expressed a high level of TNF-α *in vivo* and which reproducibly developed arthritis beginning at 4 weeks of age [6]. Interestingly, the disease in the mouse could be prevented by administration of MoAb against TNF-α. This strategy was applied to the human system. Recently, Elliot *et al.* demonstrated that the treatment with anti-TNF-α was safe and well tolerated, and that there were significant clinical and laboratory result improvements [7]. Thus, anti-TNF-α antibodies that block the interaction of the TNF-α protein and its receptor seem to inhibit endogenous cytokine function. In contrast to the *in vivo* inhibitory effect of anti-TNF-α, it has been reported that neutralizing antibodies against IL-4 function *in vivo* as a carrier that stabilizes the IL-4 protein [8], suggesting that different antibodies have different *in vivo* properties.

The level of naturally occurring autoantibodies to TNF-α is significantly increased in patients suffering from chronic infections and various inflammatory disorders, including rheumatic diseases [9]. The biological relevance of autoantibodies to TNF-α and other cytokines is unclear [10]. The aim of this study was

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to determine the function of, and epitope map the naturally occurring autoantibodies against TNF-α.

MATERIALS AND METHODS

DNA primers and synthetic peptides

Oligonucleotide primers and synthetic peptides were purchased from MedProbe A/S (Oslo, Norway) and the Public Health Research Institute (New York, NY).

Expression of the TNF-α protein in Escherichia coli *DE3 strain*

We have used the advantage of polymerase chain reaction (PCR) for expression of the mature human TNF-α protein in *E. coli* DE3 strain. A methionine (*Nco*I site) was introduced by PCR primer just before valine which is the first amino acid in the mature human TNF-α protein [11]. The gene was amplified from cDNA prepared from total RNA that was obtained from phytohaemagglutinin/lipopolysaccharide (PHA/LPS)-stimulated peripheral blood mononuclear cells (PBMC) using: primer 1, 5′AACCATGGTCAGATCATCTTCGCGAACC 3′, with a *Nco*I site; and primer 2, 5′TTGGTACCTTATCACAGGGCAATGATCCCAAA3′ with a *Bam*HI site and stop codon. The PCR product was cut with *Bam*H1 and *Nco*I restriction enzymes and then cloned into a *Bam*H1-*Nco*I-cleaved PET3d vector (Novagen, Stockholm, Sweden). The plasmid (PET3d-TNF-α) containing the human TNF-α protein with a codon for methionine at the 5′ end and stop codon at the 3′ end under T7 RNA polymerase was confirmed by DNA sequencing and then introduced into a BL21 strain. The BL21 strain contains a chromosomal copy of the T7 RNA polymerase which is under the control of the lac UV promoter [12]. The resulting strain could then be induced to synthesize the TNF-α protein by adding isopropyl-β-D-thiogalactosidopyranosid (IPTG) to the growth medium, as described previously [13].

Purification of the TNF-α protein

The recombinant TNF-α protein expressed in *E. coli* DE3 strain was analysed by 10% SDS–PAGE. The band corresponding to the 17-kD band was cut from the gel and then the protein was electroeluted.

Affinity purification of the autoantibodies

Electroeluted TNF-α protein (5 mg) was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). After blocking the active site with glycine, the TNF-α coupled Sepharose was packed into a chromatography column and extensively washed with 0·1 M glycine buffer pH 4·2 containing 0·5 M NaCl, and with 0·1 M bicarbonate buffer pH 8·3 in alternating cycles. The column was then washed with PBS pH 7·5 and then 20 ml serum from one RA patient with high titres of autoantibodies to TNF-α were applied to the column. After washing extensively with PBS, the immunoglobulins that bound to TNF-α were eluted by 0·1 M glycine–HCl buffer pH 2·5. Fractions of 50 μl were collected and neutralized. The antibodies contained in each fraction were estimated by ELISA using anti-human immunoglobulin phosphatase alkaline conjugate. Fractions with high antibody concentration were collected and concentrated after dialysis against PBS. These TNF-α eluates were further purified using protein A column. Following washing with PBS, the bound antibodies were eluted as above, neutralized and then concentrated using Centricon 30 (Amicon, Beverly, USA).

Biopanning experiments

Affinity-purified TNF-α autoantibodies (100 pg) were preabsorbed overnight with 10^{10} transducing units (TU) of the wild-type phage particles. Following preabsorption, 10^{10} TU from the nanopeptide phage library [14,15] were added and incubated for a further 12 h. Biotinylated anti-human immunoglobulin was added to the reacted library for 3 h at room temperature and the immune complexes were immobilized in streptavidin-coated polystyrene tubes. Following washing, the bound phages were eluted with acid, neutralized and then amplified in *E. coli* XL-1 cells. The amplified phages were prepared and used for a second round of biopanning experiments. As above, the amplified phages from the second round were used for a third round of biopanning.

Isolation of phage particles, DNA and sequencing

Phages from liquid culture were obtained by clearing the supernatant twice by centrifugation at 30 000 *g* for 10 min. Phage particles were precipitated with polyethylene glycol 6000/NaCl, resuspended in Tris-buffered saline (TBS; pH 7·5), filtered through 0·45-mm filter and then used for ELISA. For DNA sequencing, single-stranded bacteriophage DNA was purified and sequenced by the dideoxy chain termination method. The sequencing reactions were carried out using the Sequenase version II Kit as advised by the manufacturer (United States Biochemical, Cleveland, OH).

ELISA assays

ELISA experiments were performed as described previously [14]. Briefly, 96-well microtitre plates were coated overnight at 4°C with 50 ng of commercial TNF-α protein (Boehringer Mannheim, Mannheim, Germany) in PBS buffer/bovine serum albumin (BSA; 50 ng/well). The plates were blocked for 1 h at 37°C with PBS/0·5% BSA and then washed with PBS/Tween 20. TNF-α-binding immunoglobulins diluted in PBS/0·2% Tween/BSA (100 μl) were added to each well. After 1 h incubation at 37°C the plates were washed with PBS/Tween and incubated for 1 h at 37°C with anti-human immunoglobulin alkaline phosphatase conjugate (100 μl/well). Following further washing, substrate (*p*-nitrophenyl phosphate) was added (100 μl/well) in order to develop a colour reaction. The optical density (OD, 405 nm) of each well was measured after approximately 30 min incubation at 37°C.

In the case of TNF-α soluble receptors, plates were coated with serum's ammonium-precipitated proteins or with affinity-purified autoantibodies (1 μg/well). Following coating, the plates were washed and incubated with rabbit antibodies directed against P75 or P55 TNF-α receptors (R&D Systems, Abingdon, UK). After incubation and washing, anti-rabbit immunoglobulin alkaline phosphatase was added and then the plates were processed as above. Arbitrary results were considered positive when the absorbance was $> 1{\cdot}3\times$ the absorbance of the negative control.

Competition assays

Various concentrations of synthetic peptides were added to

glass tubes containing 100 μl of the diluted affinity-purified TNF-α autoantibodies and then incubated at 4°C overnight. After incubation, the mixtures were added onto TNF-α-coated ELISA plates. Bound autoantibodies in the presence or absence of competitor were detected as above. The inhibition of the autoantibodies to bind to TNF-α-coated plates by a competitor was expressed as the percentage of the uncompeted autoantibodies binding. All tests were performed in triplicates.

Cytotoxicity assays

The cytolytic activity of the TNF-α protein was determined on the WEHI 164 cell line. The cells were plated at 10000 cells/100 μl medium in 96-well plates. Commercial TNF-α protein (25 ng) was incubated with various concentrations of the affinity-purified autoantibodies. After incubation for 1 h at 37°C, 100 μl from each sample were added to the cells. Cells were cultured for 48 h and pulsed with 0·25 mCi ^{3}H-thymidine for the last 18 h of culture.

Immunoblotting experiments

SDS–PAGE and immunoblotting experiments were performed as described previously [13]. When anti-human immunoglobulin peroxidase conjugate was used, the colour was developed with 3,3-diaminobenzidine in sodium acetate containing 0·005% hydrogen peroxide.

Statistical analysis

All comparisons were performed using the Fisher's exact test.

RESULTS

Expression of human TNF-α protein in E. coli *DE3 strain*

The mature TNF-α protein was expressed in *E. coli* DE3 strain under the control of T7 promoter. Total extract of *E. coli* DE3 transformed by the PET3d-TNF-α contained a prominent polypeptide with mol. wt 17000 D, corresponding to the expected size of the TNF-α protein (Fig. 1a, lanes 2, 4 and 5). This protein is not visible in cells transformed with the vector PET3d (Fig. 1a, lane 3). The amount of the synthesized protein varied between the clones (Fig. 1a, lanes 2, 4 and 5). Verification of the production of biologically active TNF-α was obtained by assaying extract from *E. coli* for cytolytic activity of TNF-α on the fibrosarcoma cell line WEHI 164.

Affinity purification of TNF-α autoantibodies

TNF-α expressed in *E. coli* DE3 strain could be purified to apparent homogeneity by electroelution experiments (Fig. 1b, lane 2). Purified protein (5 mg) was conjugated to CNBr-activated Sepharose 4B and then used as support to purify the autoantibodies against TNF-α from the serum of one RA patient. This patient had high titres of autoantibodies to TNF-α as detected by immunoblotting experiments (Fig. 2a, lane 1). After removing impurities and unbound material by washing the column, the bound material was eluted by acid and then immediately neutralized. Fractions with a lot of antibodies were collected. The specificity of the eluted material was further investigated by its ability to bind the TNF-α protein. As is shown in Fig. 2b,c, the affinity-purified autoantibodies do bind to TNF-α protein in immunoblotting experiments. The identity of the heavy and light chains was further confirmed by

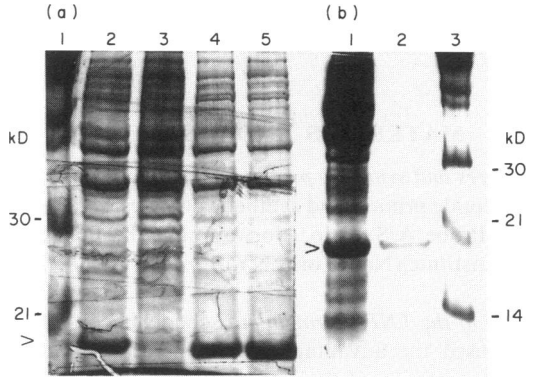

Fig. 1. SDS–PAGE of TNF-α expressed in *Escherichia coli* DE3 strain. (a) Cell extracts from *E. coli* strain expressing the TNF-α (lanes 2, 4 and 5) or not (lane 3) were analysed by 10% SDS–PAGE. The gel was stained with coomassie brilliant blue. Lane 1, Mol. wt standards. (b) Cell extracts from *E. coli* strain expressing TNF-α (lane 1) or the electroeluted TNF-α protein (lane 2) were analysed by 12·5% SDS–PAGE. Lane 3, Mol. wt standards. The arrows indicate the position of the recombinant TNF-α protein.

SDS–PAGE and immunoblotting experiments (Fig. 2d). Under reducing conditions, the eluted preparation migrated at a rate consistent with immunoglobulin heavy and light chains (lane 2). While under unreduced conditions a higher molecular band was visible (lane 1). Furthermore, a strong reactivity of anti-human immunoglobulin peroxidase conjugate with the light and heavy chains was shown in lane 3.

The autoantibodies in this patient are mainly of IgM isotype as detected by ELISA using anti-human IgM as secondary antibody.

Effect of the affinity-purified autoantibodies

A series of concentrations of the autoantibodies were used to interfere with the activity of a fixed concentration of commercial TNF-α protein sufficient to cause growth inhibition of 66% of WEHI 164 cells. The data presented in Fig. 3a,b indicate that the naturally occurring antibodies from this patient blocked the killing activity of TNF-α by 50% at 350 ng/ml. In order to check for specificity the same series of concentrations were incubated with recombinant IL-2 (rIL-2) and then assayed by the CTLL-2 assay. IL-2-induced proliferation of CTLL-2 cells was not influenced by the TNF-α-binding immunoglobulins (data not shown). A concentration of TNF-α autoantibodies alone ranging from 0 to 600 ng/ml had no effect on the WEHI 164 cell growth.

As demonstrated in Fig. 2d, the TNF-α affinity-purified autoantibodies did not contain any obvious proteins other than antibodies. However, the method used for the purification did not completely exclude the presence of TNF-α-soluble receptors. To clarify this point we have investigated the presence of TNF-α-soluble receptors in our autoantibody preparation as well as in the serum of the same patient and normal sera. Our data indicated that the amount of soluble receptors in the serum of the patient which we investigated was very low compared with positive sera. In addition, we found that the amount of soluble receptors in our TNF-α eluate preparation was similar to the background signal.

In order to rule out any possibility of the presence of soluble

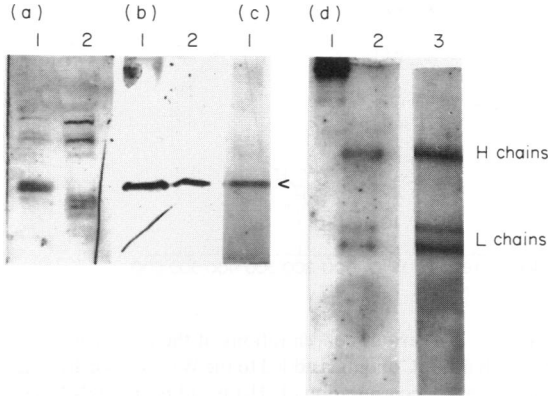

Fig. 2. Immunoblotting analysis of TNF-α protein. (a) Cell extracts from *Escherichia coli* expressing TNF-α (lane 1) or not (lane 2) were analysed with SDS–PAGE, transferred to nitrocellulose membrane, incubated with the serum (diluted 1 : 50) from one rheumatoid arthritis (RA) patient and then the immune complexes were detected as described previously [13]. (b) Cell extracts from *E. coli* expressing TNF-α induced for 60 min (lane 1) or 30 min (lane 2) were analysed by SDS–PAGE, transferred to nitrocellulose, incubated with the affinity-purified autoantibodies (200 ng/ml) and processed as in (a). (c) As (b) (lane 2), but anti-human immunoglobulin peroxidase conjugate was used as secondary antibodies. The arrow indicates the recombinant TNF-α protein. (d) SDS–PAGE and immunoblotting analysis of the affinity-purified antibodies. Affinity-purified antibodies (100 ng) were reduced by addition of β-mercaptoethanol, boiled for 3 min and then analysed by 10% SDS–PAGE (lane 2). Lane 1 as lane 2, but β-mercaptoethanol and the boiling step were omitted. The gel was stained with silver. In lane 3, separated heavy and light chains were transferred to nitrocellulose. The filter was blocked with bovine serum albumin (BSA), reacted with anti-human immunoglobulin peroxidase conjugate. After washing, bound peroxidase was developed with 3,3-diaminobenzidine in sodium acetate containing 0·005% hydrogen peroxide.

TNF-α-soluble receptors, despite their detection, the autoantibody preparation was further purified on protein A column. Protein A bind to IgG and to a lesser extent to IgM. Following elution and dialysis the antibodies were concentrated using Centricon 30 and their capacity to interfere with TNF-α bioactivity was investigated. The data presented in Fig. 3c indicated that autoantibodies against TNF-α exhibited strong neutralizing activity. These data are in accordance with the suggestion by Jeffes *et al.* [10].

Selection of bacteriophages displaying peptides that bind to TNF-α autoantibodies

To see whether the B cell response against TNF-α protein in this patient was limited or not, epitope mapping experiments were performed using a random nanopeptide phage library [15]. The selection of phages that bound to TNF-α autoantibodies was performed as described in Materials and Methods. The sequences of nanopeptides displayed by 63 random phages obtained from the third round of biopanning experiments were determined by DNA sequence analysis of the corresponding region on the PVIII gene (Table 1). Some peptides are represented more frequently in the selected library. The phages displaying these peptides were selected during the biopanning experiments, since none of them was seen in the 50 random sequenced phages from the original library. Analysis of the amino acid composition displayed by the phages that were more frequently selected revealed a consensus sequence of Ser-Ser-X, where X = Pro/Leu or Phe.

Frequency analysis of the relative occurrence of the 20 amino acids in the nanopeptides displayed by 50 random bacteriophages picked before and 63 after three rounds of selection of the phages to bind to TNF-α autoantibodies, indicated that proline ($P < 0.0003$) and serine ($P < 0.04$) were frequent in the selected phages (Fig. 4). In contrast, there was a selection against arginine ($P < 0.003$).

Specificity of autoantibody–TNF-α recognition

The specificity of the interaction of the autoantibodies with TNF-α was further assessed by inhibition experiments. Based on the peptide sequences data, three synthetic peptides (T1, T2 and T9) were synthesized and used to interfere with the binding of the autoantibodies to the commercial TNF-α-coated ELISA plates. Peptides T1, T2 and T9 significantly reduced the binding of the autoantibodies to TNF-α, whereas no inhibition was seen with an irrelevant synthetic peptide of similar length over an identical dose range (Fig. 5).

The phage library is a powerful tool for identifying ligands recognized by antibodies. The question is whether these identified ligands could induce humoral responses in different animal species. To answer this point, we investigated the immune response of mice to the phages displaying T1 and T2 peptides. The recombinant phages were shown to be useful systems for immunization [16]. Interestingly, the sera of the mice (BALB/c) immunized with phages displaying T1 and T2 peptides showed an anti-TNF-α response as detected by ELISA. This response was not found in the mice immunized with the wild type phage.

DISCUSSION

The experiments described in this study indicated that naturally occurring autoantibodies against TNF-α from one RA patient have neutralizing activity. Therefore, it may play a significant role in the regulation of TNF-α activity *in vivo*, as suggested by Jeffes *et al.* [10]. By contrast to this neutralizing activity, it was found that the TNF-α-soluble receptors stabilized the homotrimeric TNF-α molecules, and may provide a new way to increase the magnitude and duration of TNF-α effect [17]. Thus *in vivo* bio-activity of TNF-α protein seems to be regulated by opposing effects.

Mapping experiments suggested that the patient had B cell clones bearing limited specificity for the TNF-α protein, since the sequences of 63 random phages that bind to TNF-α autoantibodies displayed only nine specificities.

Normal individuals possess B lymphocytes with immunoglobulins specific for autoantigens, and it is generally thought that these B cells produce autoantibodies if they are helped by T cells [18]. The T cell help is governed by the genes in the MHC region. Thus the nature of the immune response to a particular self protein will depend on the gene products of the MHC. Therefore, other patients may have different types of autoantibodies. The observed functional response seen in tissue culture experiments may reflect the net effect of autoantibodies with different activities. This may explain the high concentration of the autoantibodies needed in order to skew the net effect towards neutralizing activity.

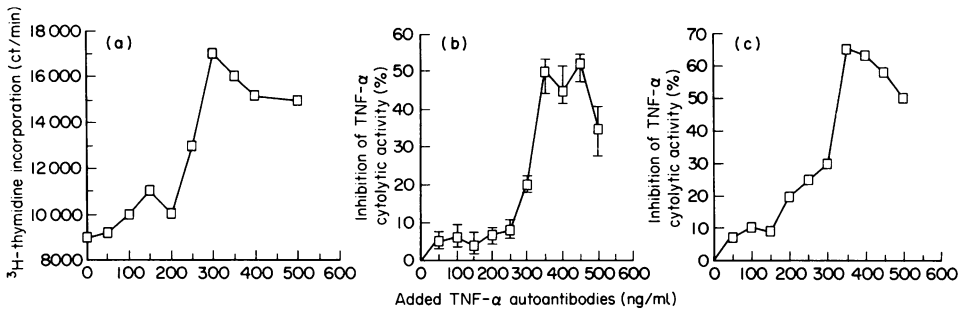

Fig. 3. Effect of the affinity-purified autoantibodies on TNF-α cytolytic activity. (a) Various concentrations of the affinity-purified autoantibodies were incubated with or without commercial TNF-α protein for 1 h at 37 °C and then added to the WEHI 164 cells. The cells were cultured for 48 h, pulsed with ^{3}H-thymidine for 18 h, harvested, and then the incorporation of ^{3}H-thymidine into DNA was determined. ^{3}H-thymidine incorporation in the control was 30 000 ct/min. (b) As (a) but represents the mean of four independent experiments expressed as per cent of inhibition. The bars indicate range values. (c) Effect of the protein A-purified autoantibodies on TNF-α cytolytic activity. The experiments were performed as in (a). Each point represents the mean of two experiments.

The epitope mapping experiments indicate that most autoantibodies recognize a conformation-dependent epitope involving the amino acids serine and proline (Fig. 4 and Table 1). Combination of Ser-Pro occurs five times in TNF-α proteins, compared with, for example, zero times in the IL-2 protein. The peptide sequence, T2, contains Ala-X-X-Leu-Leu-Ala corresponding to amino acids 33 and 36–38 on the TNF-α protein [11]. The selection of this particular epitope could be due both to the presence of autoantibodies against amino acids 33–38 in TNF-α protein or a conformational epitope involving all amino acids. The T9 peptide sequence contains Pro-X-X-Lys-Pro-Val corresponding to residues 8 and 11–13 in the TNF-α protein. Interestingly, residues 11–13 are involved in the binding site of TNF-α to its receptors [19].

Comparison of the frequency of the 20 amino acids in the phages selected to bind to TNF-α autoantibodies (Table 1) with 50 random sequences from the original library indicates that there was a selection for proline, serine and to lesser extent leucine (Fig. 4). In contrast, there was a selection against arginine.

A different profile of selection was seen when the same phage library was used to map a MoAb [15]. Indeed, frequency analysis of the phages selected by Felici *et al.* to bind to the MoAb directed to the peptide VQGEESNDK, present in IL-1β protein revealed SND, which is the consensus sequence of the epitope of the MoAb. Furthermore, we have found that the frequency of occurrence of each amino acid in the peptide sequences of 50 random phages that bind to antibodies from synovial fluid obtained from one RA patient is different from the phages that bind to TNF-α autoantibodies (Dybwad *et al.*, manuscript in preparation). Thus our data indicate that there is a clear selection of the phages that bind to TNF-α autoanti-

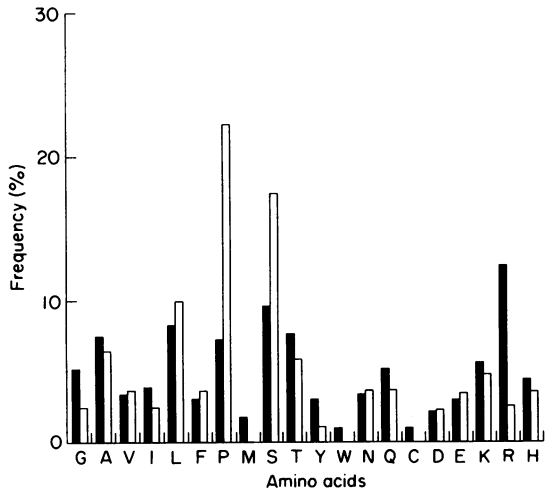

Fig. 4. Relative abundance of the 20 amino acids in the nanopeptide displayed by the 50 unselected (■) and 63 selected phages to bind to human TNF-α autoantibodies (□). The figure shows the frequency of occurrence of each amino acid calculated as the number observed divided by the total residues in the sequenced peptides.

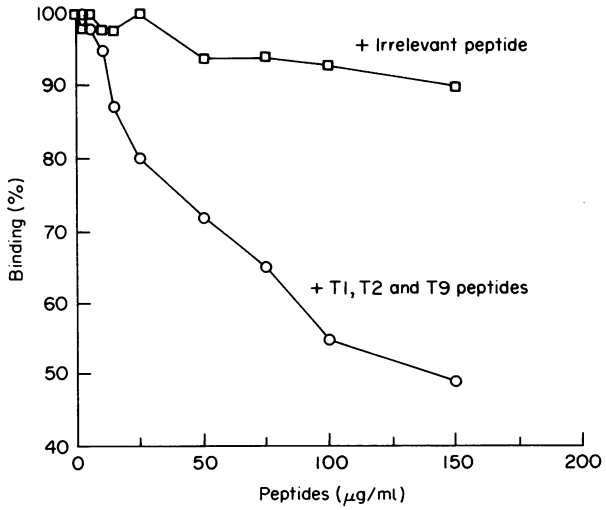

Fig. 5. Inhibition of the autoantibodies to bind to TNF-α protein by synthetic peptides. Various concentrations of a pool of equal parts of the T1, T2 and T9 peptides (Table 1) or the control peptide (ADGGAQGTA) were incubated overnight at 4°C with the same concentration of the autoantibodies. Following incubation the binding was analysed by ELISA on plates coated with commercial TNF-α protein, and then the residual binding was calculated.

Table 1. Amino acid sequences of the nanopeptides displayed by 63 random bacteriophages picked after selection for the ability to bind to human TNF-α autoantibodies

	Sequences	Frequency
T1	Asn-Ser-Ser-Pro-Tyr-Leu-Asn-Thr-Lys	20
T2	Ala-Ser-Ser-Leu-Leu-Ala-Ser-Ser-Pro	16
T3	Pro-Gln-Ser-Pro-Gly-Ser-Ser-Phe-Pro	10
T4	Pro-His-Asn-Arg-Gln-Glu-Ser-Pro-Ala	7
T5	Ile-Pro-Phe-Pro-Thr-Leu-Phe-Ala-Pro	2
T6	Pro-His-Glu-Ser-Asp-Ala-Thr-Val-Arg	2
T7	Leu-Val-Gly-Thr-Pro-Gln-Lys-Thr-Lys	2
T8	Pro-Ser-Pro-Ser-Leu-Ser-His-Pro-Leu	1
T9	Pro-Pro-Leu-Lys-Pro-Val-Ile-Asp-Glu	1

bodies, and involvement of the selected amino acids proline, serine and to a lesser extent leucine, in the binding of the phages to the autoantibodies. The selection of the phage to bind to TNF-α autoantibodies was further confirmed by the fact that the sera of mice (BALB/c) immunized with phages displaying ASSLLASSP and NSSPYLNTK peptide showed an anti-TNF-α response, as detected by ELISA. This response was not found in mice immunized with the wild type phage. These data confirm that the selected phages are specific for antibodies that are directed against TNF-α protein. In addition, these preliminary data indicated that the recombinant phages selected from the phage libraries could be used as carriers for immunization, and therefore as a tool for vaccine development.

Whether these autoantibodies against TNF-α arise from molecular mimicry or are directed to TNF-α protein is not clear, and needs more investigation. More recently Griffiths *et al.* demonstrated that it is possible to pick up self-reactive autoantibodies against TNF-α encoded by somatically or unsomatically mutated V genes from peripheral blood lymphocytes from unimmunized humans [20]. Thus, B cells secreting autoantibodies to TNF-α could be derived from virgin B cells. Our study suggests that the patient had B cell clones bearing limited specificity for TNF-α.

Finally, the use of the phage library technique to investigate the nature of B cell responses to known antigens may permit direct correlation between B cell response and antigen specificity. In future this may help to understand the immune response to any protein and to search for protective antibody ligands which could boost protective immunity. Indeed, using a new nanopeptide phage library with more complexity than the library used in this study, we are able to select ligands from the serum that are similar to the peptide used for immunization (Dybwad *et al.*, manuscript in preparation).

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REFERENCES

1 Arai KI, Lee F, Miyajima A. Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem 1990; **59**:783–836.
2 Schattner A. Lymphokines in autoimmunity—a critical review. Clin Immunol Immunopathol 1994; **70**:177–89.
3 Lipsky PE, Davis LS, Cush JJ *et al.* The role of cytokines in the pathogenesis of rheumatoid arthritis. Springer Semin Immunopathol 1989; **11**:123–62.
4 Deleuran BW, Chu CQ, Field M *et al.* Localization of tumor necrosis factor receptors in the synovial tissue and cartilage–pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1992; **35**:1170–8.
5 Williams RO, Feldman M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 1992; **89**:9784–8.
6 Keffler J, Probert L, Cazlaris H *et al.* Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J 1991; **10**:4025–31.
7 Elliot MJ, Maini RN, Feldmann M *et al.* Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor α. Arthritis Rheum 1993; **36**:1681–90.
8 Finkelman FD, Madden KB, Morris SC *et al.* Anti-cytokine antibodies as carrier proteins. Prolongation of *in vivo* effects of exogenous cytokines by injection of cytokine–anti-cytokine antibody complexes. J Immunol 1993; **151**:1235–44.
9 Fomsgaard A, Svenson M, Bendtzen, K. Auto-antibodies to tumour necrosis factor alpha in healthy humans and patients with inflammatory diseases and Gram-negative bacterial infections. Scand J Immunol 1989; **30**:219–23.
10 Jeffes EWB, Ininns EK, Schmitz KL *et al.* The presence of antibodies to lymphotoxin and tumor necrosis factor in normal serum. Arthritis Rheum 1989; **32**:1148–52.
11 Pennica D, Nedwin GE, Hayflick JS *et al.* Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984; **312**:724–9.
12 Studier F, Moffatt B. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986; **189**:113–30.
13 Sioud M, Drlica K. Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme. Proc Natl Acad Sci USA 1991; **88**:7303–7.
14 Dybwad A, Førre Ø, Kjeldsen-Kragh J *et al.* Identification of new B cell epitopes in the sera of rheumatoid arthritis patients using a random nanopeptide phage library. Eur J Immunol 1993; **23**:3189–93.
15 Felici F, Castagnoli L, Musacchio AJ *et al.* Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. J Mol Biol 1991; **222**:301–10.
16 Cruz VF, Lal AA, McCutchan TF. Immunogenicity and epitope mapping of foreign sequences via genetically engineered filamentous phage. J Biol Chem 1988; **263**:4318–22.
17 Aderka D, Engelmann H, Maor Y *et al.* Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med 1992; **175**:323–9.
18 Casali P, Notkins AL. Probing the human B-cell repertoire with EBV: polyreactive antibodies and $CD5^+$ B lymphocytes. Ann Rev Immunol 1989; **7**:513–35.
19 Eck MJ, Sprand SR. The structure of tumor necrosis factor-alpha at 2.6 Å resolution. Implications for receptor binding. J Biol Chem 1989; **264**:17595–605.
20 Griffiths AD, Malmqvist M, Marks JD *et al.* Human anti-self antibodies with high specificity from phage display libraries. EMBO J 1993; **12**:725–34.