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ORIGINAL INVESTIGATION

**Klaus Ritter · Annette Fudickar · Nicola Heine
Reiner Thomssen**

Autoantibodies with a protective function: polyreactive antibodies against alkaline phosphatase in bacterial infections

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Abstract In patients with acute bacterial infections antibodies directed against a particular bacterial antigen were detected. The molecular mass of this bacterial antigen was 50 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By comparison of the NH_2-terminal amino acid sequence, the 50-kDa antigen was identified as alkaline phosphatase (AP). Affinity-purified antibodies from patients' sera directed against the bacterial AP (anti-alpha) were also shown to react with human and animal AP, which have different structures. Anti-alpha are IgG subtype 3 immunoglobulins, and their light chains are of the kappa type. Upon isoelectric focussing, the anti-alpha formed a scalariform pattern with five to seven bands in the pH range 7–9. The anti-alpha have an opsonic activity and cause a five- to eightfold increase of phagocytosis of gram-positive and gram-negative bacteria. According to their polyreactivity, their sudden rise early in infection, their oligoclonality, as well as their opsonizing properties, they are assumed to be permanently available natural antibodies that take part in early defence mechanisms.

Key words Autoantibodies · Alkaline phosphatase · Acute bacterial infections

Introduction

Acute bacterial infections are often accompanied by a polyclonal B cell stimulation. This is induced by lipopolysaccharides of gram-negative bacteria or by peptidoglycan of gram-positive bacteria [1]. The unspecific B cell stimulation causes a considerable rise of serum immunoglobulins of both isotype G and M. However, little is known about the specificity of the antibodies. In some bacterial infections heterophilic antibodies against the Forssman-antigen from sheep [2] or against the bovine cardiolipin [3] were detected. These antibodies seem to have no effect on the clinical course of infection. In contrast, autoreactive cold agglutinins with specificitiy for the blood group antigen 'I' may lead to hemolysis in mycoplasma infections [4]. Recently, cationic glycoproteins were detected in cerebrospinal fluid (CSF) and sera of patients with acute bacterial infections [5]. We isolated these substances from sera of patients in the early phase of bacterial infections and identified them as class G immunoglobulins [6]. The aim of this study was to clarify the specificity of the antibodies and to elucidate their function in acute bacterial infections.

K. Ritter (✉)
Institute of Medical Microbiology, RWTH Aachen,
Pauwelsstrasse 30, D-52074 Aachen, Germany
Fax: 49-241 8888483

A. Fudickar · N. Heine · R. Thomssen
Department of Medical Microbiology,
University of Göttingen, Kreuzbergring 57,
D-37075 Göttingen, Germany

Materials and methods

Sera

Sera from patients with acute bacterial infections and sera from healthy controls were investigated.

Preparing the antigens

The bacterial strains *Escherichia coli*, *Neisseria meningitidis, Streptococcus (Str.) pyogenes,* and *Staphylococcus (Staph.) aureus* isolated from clinical specimens submitted to the diagnostic section of the department were used. The bacterial cells were harvested by centrifugation and washed three times in 0.9% NaCl. The pellet (100 mg packed cells) was suspended in 100 µl 0.9% NaCl (w/v). Crystalline lysozyme in the concentration 1 mg/g bacteria was added, and lysis was allowed to proceed for 2 h at 30°C. Afterwards equal volumes of lysis buffer [7] containing 9.5 M urea, 2% (w/v) Nonidet-P40 and 5% (v/v) 2-mercaptoethanol were added to the cell suspension; further disintegration was achieved by freezing and thawing. The cell lysates were centrifuged at 10,000 *g* for 5 min. The supernatant fluid was used for antigen preparation by SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting.

Polyacrylamide gel electrophoresis

Composition and electrophoresis of SDS-polyacrylamide slab gels were carried out as described by Gultekin and Heermann [8]. The electrotransfer of proteins from SDS gels to polyvinylidenedifluoride membranes (Millipore Continental Water Systems, Bedford, Mass.) was performed by transversal electrophoresis [8]. Gel preparation for isoelectric focusing (IEF) was carried out as described by Allen [9]. IEF was achieved as described by Schipper et al. [10]. Afterwards Western blotting was carried out according to the method of Bowen applying pressure-diffusion blotting [11]. The polyacrylamide gels were stained with Coomassie blue.

Isolation and purification of p50

P50 was isolated from an *E. coli* cell lysate by means of electroelution [12] following preparative SDS-PAGE on a 13% gel. After further purification on a SDS-10–18% gradient polyacrylamide gel, the electrotransfer on PVDF was performed for protein sequencing.

Immunostaining of the blots

Horseradish peroxidase-conjugated antibodies specific for Ig/H and Ig/L chains (Dako, Denmark) were used in the dilution 1:1,000 in phosphate-buffered saline (PBS: 2 mM KH_2PO_4, 180 mM NaCl, 9 mM $Na_2HPO_4 \cdot 2\ H_2O$, pH 7.2) containing 20% (v/v) fetal calf serum.

Affinity purification of anti-alkaline phosphatase antibodies and determination of IgG subclasses by radial immunodiffusion

Purification of antibodies against alkaline phosphatase from patients' sera was performed by the method described for anti-TPI antibodies [13]. For determination of the IgG subclasses, a commercially available radial immunodiffusion kit was applied according to the producer's instructions (ICN Biomedicals, Meckenheim, Germany).

Determination of the amino acid sequence

To determine the NH_2-terminal amino acid sequence, an automatic gas-phase sequencer (470 A; Applied Biosystems, Inc., Foster City, Calif.) was used [14]. The amino acid sequence obtained was compared with data of the protein sequence data bases of the Martinsried Institute for Protein Sequences (Martinsried, Germany) and the Protein Identification Resource (Atlanta, Ga.).

Monocyte preparation

Heparinized venous blood from healthy donors was diluted 1:1 (v/v) with RPMI 1640, put on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient, and centrifuged at 400 *g* for 45 min at 20°C. The mononuclear band obtained was washed three times in RPMI 1640 with 10% fetal calf serum (FCS), the cells were counted, resuspended in culture medium, and dispensed into 24-well flat-bottom plates. After incubation for 4 h at 37°C in a 5% CO_2 atmosphere, nonadherent cells were removed, and fresh culture medium was added. Approximately 90% of the adherent cells were monocytes that stained with α-naphthyl acetate esterase.

Labeling of bacteria

After separation from the culture medium *E. coli* and *Str. pyogenes* (10^7 cells per sample) were resuspended in RPMI 1640 containing 3.7 MBq ^{51}Cr (Amersham-Buchler, Braunschweig, Germany). To remove the radioactive chromium which had not been taken up, bacteria were washed in RPMI 1640 medium after a 4-h incubation period.

Opsonization of bacteria

The ^{51}Cr-labeled bacteria were incubated for 1 h with affinity-purified anti-alpha in the concentration of 1.44–57.6 µg/ml or with specific antisera directed against *E. coli* OK, respectively, *Str. group* A (Biotrend, Cologne, Germany) and allowed to opsonize. For control, human IgG of irrelevant specificity from a patient with a plasmacytoma was used. After opsonization and immediately before their addition to phagocytes, the bacteria were washed and resuspended in RPMI 1640 with 10% heat-inactivated FCS.

Phagocytosis assay

The human monocytes were incubated for 2 h at 37°C with ^{51}Cr-labeled preopsonized as well as untreated bacteria in the ratio 100 bacteria to 1 monocyte. After the incubation period free bacteria were removed in several washing steps using cold Hanks' solution. The monocytes were further incubated for 45 min at 37°C in RPMI 1640 supplemented with 5 µg gentamicin/ml to kill extracellular bacteria. Subsequently, the monolayers were washed three times with Hanks' solution. The monocytes were lysed with a 5% saponin solution. The rate of phagocytosis was evaluated by the ^{51}Cr uptake of the monocytes. Each assay was done in quadruplicate. The ratio of phagocytosis was calculated as: $100 \times (cpm_{sample}/cpm_{control})$.

Results

Humoral immune reaction against bacterial p50

In the early phase of acute bacterial infections antibodies of the immunoglobulin class G (IgG) appeared in patients' sera that reacted with a component of the lysed bacterial cell. A typical example is shown in Fig. 1. IgG antibodies from the serum of a patient with sepsis caused by *E. coli* react with an 50-kDa antigen of *E. coli* in immunoblot (Fig. 1, lane 1). This antigen, p50, also occurs in other gram-negative bacteria such as *N. meningitidis* and also in gram-positive bacteria such as *Str. pyogenes* and *Staph. aureus*.

Identification of p50

After separation of the *E. coli* cell lysate on preparative SDS gels a sufficient amount of purified p50 antigen for further analysis was obtained by electroelution. The protein was identified by using NH_2-terminal amino acid sequence analysis. The sequence of the 28 NH_2-terminal

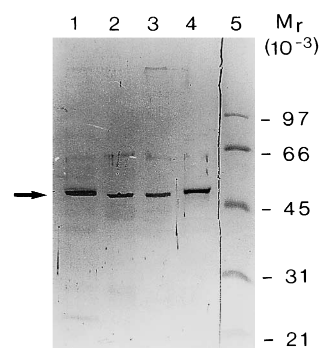

Fig. 1 Immunoblot. Reaction of antibodies from the serum of a patient with *Escherichia coli* septicemia with bacterial lysates after separation by SDS-PAGE. *Lane 1* Lysate from *E. coli*; *lane 2* lysate from *Streptococcus pyogenes*; *lane 3* lysate from *Staphylococcus aureus*; *lane 4* lysate from *Neisseria meningitidis*. Second antibody: peroxidase-labeled anti-human IgG; *lane 5* molecular mass marker

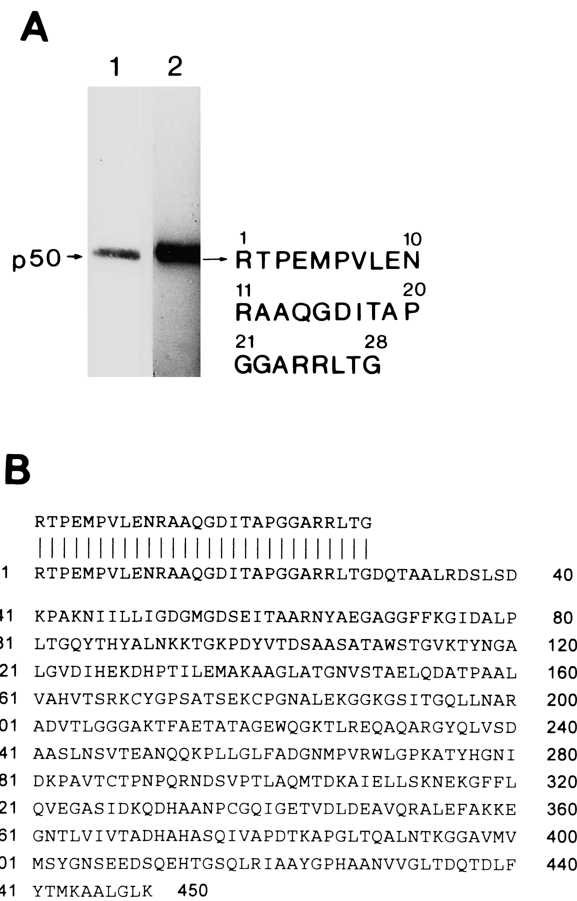

Fig. 2A, B Identification of p50. **A** *Lane 1* Purified p50 (immunoblot); *lane 2* purified p50 (Coomassie blue and NH_2-terminal amino acid sequence). **B** Comparison of NH_2-terminal amino acids of p50 and alkaline phosphatase from *E. coli*; one letter code was used. These sequence data are available from EMBL/GenBank/DDBJ under accession number X04586

amino acids of p50 is shown in Fig. 2A. This sequence was compared with data of the Martinsried Insitute for Protein Sequences and the Protein Identification Resource (Fig. 2B). There was complete identity with a sequence from the alkaline phosphatase from *E. coli* [15].

To confirm that IgG anti-p50 recognizes alkaline phosphatase, SDS-PAGE and Western blots were performed with a commercially available alkaline phosphatase preparation gained from *E. coli*. The IgG fraction of all anti-p50-positive sera from patients with acute bacterial infections reacted with the alkaline phosphatase from *E. coli*

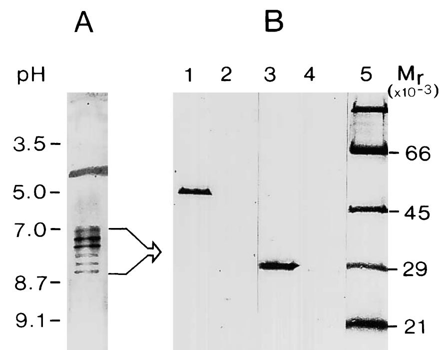

Fig. 3 **A** Isoelectric focusing blot of affinity-purified anti-alpha: reaction with alkaline phosphatase from *E. coli*. **B** SDS-PAGE from affinity-purified anti-alpha and immunoblot; *lane 1* anti-human Ig/H γ; *lane 2* anti-human Ig/H μ; *lane 3* anti-human Ig/L κ; *lane 4* anti-human Ig/H λ; *lane 5* molecular mass marker

(data not shown), showing that the antibodies possess specificity for alkaline phosphatase. The antibodies were termed anti-alpha.

Characterization of anti-alpha

Anti-alpha was purified from the sera of ten patients with acute bacterial infections by affinity chromatography. After IEF, affinity-purified anti-alpha showed five to seven bands in the range pH 7–9. Each of these bands possessed a specificity for alkaline phosphatase, as shown in Fig. 3A. Figure 3B shows that anti-alpha possess the Ig/H chain γ and the Ig/L chain κ. In radial immundiffusion anti-alpha were recognized as IgG subclass 3 (IgG3) (data not shown).

Affinity-purified IgG anti-alpha was not only specific for bacterial alkaline phosphatase, but also reacted with human alkaline phosphatase from various tissues, and with animal alkaline phosphatase as presented in Table 1. Hyperimmune sera from rabbit, in contrast, did not react with bacterial alkaline phosphatases, neither did monoclonal antibodies from *E. coli* recognize animal alkaline phosphatases.

Biological effect of anti-alpha

To clarify the significance of anti-alpha for the course of bacterial infections, the influence of the antibodies on phagocytosis of bacteria was examined as shown in Table 2.

Table 1 Reaction of anti-alpha and hyperimmune sera with various alkaline phosphatases of different origin

Antibodies	Antigen: alkaline phosphatase				
	Placenta (human)	Intestine (bovine)	Liver (bovine)	Intestine (porcine)	*E. coli* (bacterial)
Anti-alpha	+	+	+	+	+
Rabbit anti-AP (to bovine, intestinal)	+	+	+	+	–
Monoclonal anti-AP (to *E. coli*)	–	–	–	–	+

Table 2 Effect of anti-alpha on phagocytosis of *Escherichia coli* and *Streptococcus pyogenes* by human macrophages. The values presented are means of quadruplicate testing (*n.d.* not done)

	Antibody concentration (μg/ml)	*E. coli*		*Str. pyogenes*	
		^{51}Cr uptake of macrophages (cpm)	Phagocytosis rate (%)	^{51}Cr uptake of macrophages (cpm)	Phagocytosis rate (%)
Anti-alpha	0	516	14	349	11
	1.44	1126	31	706	23
	5.76	2140	59	1118	36
	14.4	2544	70	2543	83
	57.6	2561	70	2604	85
Irrelevant IgG (plasmacytoma)	10	530	15	288	9
Anti-*E. coli* (from rabbit)	3.5	3654	100	n.d.	n.d.
Anti-*Str.* group A (from goat)	4.0	n.d.	n.d.	3071	100

Incubation of the bacteria with anti-alpha led to a rise of the phagocytosis rate depending on the antibody concentration. For *E. coli* a fivefold rise compared to non-opsonized bacteria was measured after the addition of 15 μg/ml anti-alpha. This was 70% of the phagocytosis rate gained by opsonization with specific antibodies. Phagocytosis of *Str. pyogenes* increased almost eightfold in the presence of anti-alpha; addition of 15 μg/ml anti-alpha caused a rise in phagocytosis to 83% of the rate that could be achieved by *Str. pyogenes*-specific antibodies. Phagocytosis induced by anti-alpha up to a concentration of 15 μg/ml anti-alpha was dose dependent; higher anti-alpha concentrations did not improve phagocytosis (Table 2).

Whereas maximal phagocytosis of *E. coli* and *Str. pyogenes* was achieved by opsonization with specific antibodies, only a low rate of phagocytosis of 11% and 14%, respectively, was obtained for non-opsonized *E. coli* and *Str. pyogenes*. Addition of human IgG of irrelevant specificity did not lead to a significant rise of phagocytosis.

Discussion

In the early phase of bacterial infections antibodies become detectable in patients' sera that belong to the IgG class and recognize an antigen of 50 kDa (p50) that is present in gram-positive and gram-negative bacteria. By NH_2-terminal sequencing p50 was identified as AP. The affinity-purified antibodies against bacterial AP, termed here anti-alpha, also reacted with AP from human and animal tissue. Thus, anti-alpha possess three features: (1) they are adaptive antibodies, which are directed against bacterial AP, (2) they are heterophilic antibodies that react with AP of animal origin, and (3) they are autoantibodies, as they react with human AP. Since AP of different organs, tissues, and species vary considerably in their structure [15–17] anti-alpha may be regarded as polyreactive autoantibodies.

Polyreactivity is a property of natural antibodies. Indeed, natural autoantibodies against intestinal AP have been recently detected in human sera [18]. These natural autoantibodies are of low avidity, are polyreactive and cross-react with myelin basic protein, S-100 protein, transferrin, and myosin [18]. Although no specificity for bacterial AP or liver AP was found [18], it cannot be excluded that anti-alpha-producing B cells derive from this pool of B cells, which expand due to their contact with bacterial AP. The oligoclonality of anti-alpha is indicated by the narrow pattern of only a few bands in IEF, the restriction to Ig/L chain κ and the subclass restriction to IgG3. Acute infections have been shown to induce natural autoantibodies that are oligoclonal and restricted to a certain immunoglobulin subclass [19, 20].

Natural autoantibodies are part of a strictly regulated immunological network that on the one hand probably prevents autoaggression [21], and on the other hand seems to play an important role in the first line of defense against infections [22]. Acute viral or bacterial infections may disturb this network to such an extent that B cell clones producing natural autoantibodies may expand beyond control. In some conditions autoantibodies of a certain specificity may gain pathogenic significance and prolong the course of infection [23, 24]. Anti-alpha, in contrast, seem to have a protective effect. They bind to bacteria and promote phagocytosis. In many species of bacteria, AP are found in the cytoplasm and in the periplasmatic space [25]. As bacteria are able to release AP into the extracellular space [26], AP also reach the outer membrane of gram-negative bacteria [27] or the outer layer of the cell wall. By binding to these AP molecules the anti-alpha act as opsonins that lead to an increase in phagocytosis of bacteria.

Macrophage-induced phagocytosis is important for the local limitation and control of the bacterial infection. Opsonizing antibodies may augment phagocytosis, or in encapsulated strains of bacteria only render phagocytosis possible [28]. Unless there is a reinfection bacteria-specific antibodies are not present at the beginning of the infection. Before the ocurrence of the specific immune response bacteria may induce the production of natural autoantibodies with specificity for an antigen that is also widespread in bacteria, such as AP. Thus, anti-alpha seems to play an important role in the early defense against ba-

cerial infections. Being IgG3 antibodies anti-alpha are not only strong opsonins and highly macrocytophilic, they are also not influenced by bacterial virulence factors such as the Fc-receptor type I on staphylococci or the Fc-receptor type II on group A streptococci [29].

From these findings we conclude that anti-alpha are probably part of a defense mechanism that has emerged early in ontogenesis and that is of importance for the control of bacterial infections. If further investigations can exclude any pathogenic effect of anti-alpha on eukaryontic cells, anti-alpha may prove to be of use in supportive therapy, for example for local infections.

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