

Manganese Superoxide Dismutase as a Target of Autoantibodies in Acute Epstein-Barr Virus Infection

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Summary

Antibodies directed against the autoantigen p26 were detected in sera from 32 patients with acute Epstein-Barr virus (EBV) infection and clinical symptoms of infectious mononucleosis. P26 has now been identified as the enzyme manganese superoxide dismutase (MnSOD) by comparison of the NH₂-terminal amino acid sequence. Antibodies against MnSOD belong to the immunoglobulin class M. They are not detectable in sera of patients with other herpesvirus infections. In the 32 patients investigated, the rise and fall of the autoantibodies coincides with the clinical symptoms. In vitro, the autoantibodies were shown to inhibit the dismutation of superoxide radicals by blocking MnSOD. As presented in the discussion this effect may contribute to the pathogenesis of acute EBV infection.

Acute Epstein-Barr virus (EBV) infection becomes apparent with the clinical picture of infectious mononucleosis (IM). Due to its B lymphotropism, EBV is not only capable of infecting and immortalizing B cells, but is also capable of activating resting B cells, which leads to the production of antibodies of various specificities including heterophil antibodies, cold agglutinins against blood group antigens, as well as antibodies reacting with the cytoskeleton, and nuclear antigens (1). We discovered two autoantibodies against the cellular antigens p26 and p29 (2). The antigen p29 was identified as human triosephosphate isomerase (TPI) as presented previously (3). Anti-TPI was shown to be a causative agent for hemolysis, which is a complication of acute EBV infection (4, 5). With a new approach, we were able to identify the autoantigen p26 as manganese superoxide dismutase (MnSOD).

Materials and Methods

Sera. Sera from 32 patients with acute EBV infections and sera from 25 patients with other herpesvirus infections were investigated (10 acute varicella-zoster infections, 9 cytomegalovirus infections, 6 herpes simplex virus infections). Sera from 50 healthy volunteers were tested as controls.

Preparing the Antigens. Various EBV genome positive and negative lymphoblastoid cell lines (0.1 ml packed volume) were resuspended in equal volumes of lysis buffer (6) containing 9.5 M urea, 2% (wt/vol) NP-40, and 5% β -mercaptoethanol; further disintegration was achieved by five cycles of freezing and thawing (-25°C /room temperature). The cell lysates were centrifuged at 10,000 g for 5 min. The supernatant fluid was used for antigen preparation by SDS-PAGE followed by immunoblotting.

PAGE. Composition and electrophoresis of SDS-13% (wt/vol)

polyacrylamide slab gels were carried out as described by Heermann et al. (7). The electrotransfer of proteins from SDS gels to polyvinylidenedifluoride (PVDF) membranes (Millipore Continental Water Systems, Bedford, MA) was performed by transversal electrophoresis (8). Nonreducing SDS-PAGE was carried out using the method of Maurer (9) with minor modifications. Staining of the SDS-polyacrylamide gels was done with Coomassie blue or silver salts (10).

Isolation and Purification of p26. p26 was isolated from a Raji cell lysate by means of electroelution (11) after preparative SDS-13% PAGE. After further purification on SDS-10–18% gradient polyacrylamide gel, the electrotransfer on PVDF for protein sequencing was performed.

Immunostaining of the Blots. Horseradish peroxidase-conjugated antibodies specific for Ig/H and Ig/L chains (DAKO, Glostrup, Denmark) were used in a dilution of 1:1,000 in PBS (2 mM KH₂PO₄, 180 mM NaCl, 9 mM Na₂HPO₄·2 H₂O, pH 7.2), containing 20% (vol/vol) fetal calf serum.

Affinity Purification of Anti-MnSOD Antibodies. Purification of autoantibodies was performed as described for anti-TPI antibodies (12).

Incomplete Proteolytic Cleavage of p26. Purified p26 was cleaved with endoproteinase Glu-C (Protease V 8) from *Staphylococcus aureus* V8 (Boehringer Mannheim, Mannheim, Germany) according to the method of Houmard and Drapeau (13). However, cleavage of p26 was not completed to gain fragments of a sufficient size to be recognized in immunoblot of IM sera after SDS-PAGE separation.

Determination of Amino Acid Sequence. To determine the NH₂-terminal amino acid sequence, an automatic gas phase sequencer (model 470 A; Applied Biosystems, Inc., Foster City, CA) was used (14). The amino acid sequence obtained was compared with data of the protein sequence databases of the Martinsried Institute for Protein Sequences (Martinsried, Germany) and the Protein Identification Resource (Atlanta, GA).

Isolation of MnSOD from Liver Tissue. MnSOD was purified

from 75 g human normal liver tissue obtained at the autopsy of a patient who did not have neoplastic or hepatic diseases according to the procedure described by Matsuda et al. (15). Activity was visualized on 10% polyacrylamide gels under nondenaturing conditions (16).

Inhibition of the Enzyme Activity of MnSOD In Vitro by Antibodies against p26 (MnSOD). The inhibitory activity of anti-MnSOD antibodies in MnSOD was determined by applying the method Yoshida and Miwa (17) used for phosphoglycerate kinase. Briefly, the autoantibodies were adjusted to 2 $\mu\text{g}/\text{ml}$ in 0.15 M NaCl. 20 μl MnSOD (833.5 nkat/ml) were added to 50 μl antibody solution (0.2 $\mu\text{g}/\text{ml}$). After incubation for 18 h at 4°C, the MnSOD activity was measured using the optic assay described by McCord and Fridovich (18). Each assay was done in quadruplicate.

Results

The IgM-fraction from sera of patients with IM reacted with lymphoblastoid cell lines and primary lymphocytes, and was exhibited as a weak granular cytoplasmatic fluorescence, which was more dense around the nucleus than in the periphery. To identify the cellular antigens, various types of human lymphocytes including Raji cells were lysed. The lysates were separated in SDS-PAGE. After electrotransfer on a PVDF membrane, the lysates were used for immune reactions with patients' sera. Two major antigens were recognized in all cell lysates. In Fig. 1, four examples of the typical reaction of sera from the acute phase of an acute EBV infection on a Raji cell lysate are shown. IgM antibodies react with two proteins of a molecular mass of 26 (p26) and 29 kD (p29), respectively. We had previously identified p29 as TPI. IgM antibodies to p26/p29 (TPI) were not detected in the sera of healthy probands or of patients with acute herpes simplex virus or varicella-zoster virus infections. Only a weakly positive reaction against p26/p29 (TPI) was observed in the serum of a patient with an acute cytomegalovirus infection and a reactivated EBV infection.

After separation of the Raji cell lysate in preparative SDS gels, a sufficient amount of purified p26 could be gained by electroelution. Due to NH_2 -terminal blocking, the protein identification of p26 by amino acid sequencing was not successful. After incomplete cleavage of p26 with V8 protease, the fragments were separated in SDS-18% PAGE. Their reaction with the serum of patient with IM was tested by immunoblot. As Fig. 2 A demonstrates, a peptide of 15 kD (p15) gave a positive reaction. The sequence of the 18 NH_2 -terminal amino acids of this p15 fragment was determined with a gasphase sequencer. The sequence of this peptide was compared with data of the protein sequence databases of the Martinsried Institute for Protein Sequences and the Protein Identification Resource (cf 2B). There was complete identity with a sequence part of human MnSOD (19). The NH_2 terminus of the 15-kD fragment starts with alanine in accordance with position 72 of MnSOD. Next to it in position 71 there is glutamic acid, the cleavage point of the V8 protease. Because of its size, the 15-kD fragment probably extends to the glutamic acid in position 211, a further cleavage point of the V8 protease. Thus, it is in accordance with the sequence of MnSOD (19).

MnSOD was isolated from human liver tissue in order to

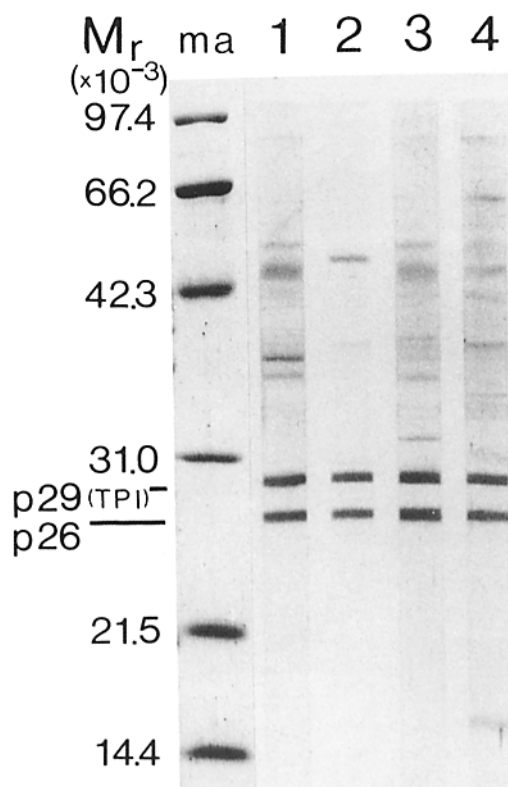


Figure 1. Reaction of sera from four patients in the acute phase of EBV infections on Raji cell lysate after SDS-PAGE. (Immunoblot, detection of IgM isotype); ma, molecular mass marker.

test whether anti-p26 antibodies from patients' sera recognize MnSOD. In native PAGE, the purified enzyme showed up at about 100 kD, as was demonstrated by silver staining in Fig. 3, lane a. Contaminations with other liver proteins were not detected. The protein of the 100-kD band was enzymatically active (cf 3 b), and was recognized by the antibodies from patient's sera (cf 3 c). In the denaturing SDS-PAGE the monomers of the tetrameric MnSOD molecule were found at 26 kD (cf 3 d). This band is also recognized by the antibodies (cf 3 e).

Antibodies against MnSOD were purified from the sera of four patients with IM. The concentration of the affinity purified antibodies was adjusted to 0.2 $\mu\text{g}/\text{ml}$. All of the four affinity purified antibodies inhibited the activity of MnSOD as shown in Fig. 4. However, the inhibitory activity varied considerably. Antibodies from patient no. 1 had the weakest effect, leading to a reduction of the enzyme activity from 833.5 nkat/ml to 542 nkat/ml, which is a 35% inhibition. The strongest inhibition was obtained by the antibodies of patient no. 3; reaching 89% by reducing the enzyme activity to 91.5 nkat/ml (cf 4). With 71 and 57% inhibition of MnSOD, the efficacy of antibodies from patient nos. 2 and 4 was between the above mentioned. Patient no. 3, whose anti-MnSOD antibodies showed the strongest inhibitor activity, was suffering from a prolonged course of the illness with distinct hepatitis and splenomegaly. Patient no. 2 had been admitted to hospital with a rash and suspected meningitis. In the 32 patients investigated the IgM anti-MnSOD

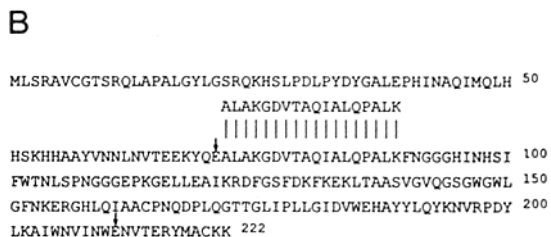
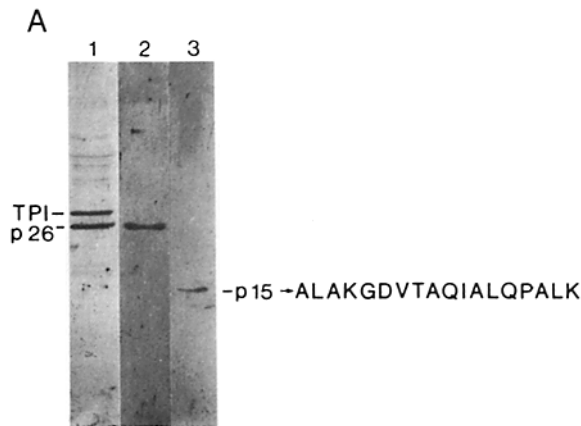


Figure 2. Identification of p26. (A) Lane 1, Raji cell lysate (immunoblot); lane 2, purified p26 (immunoblot); lane 3, p15-fragments from purified p26 after V8 protease cleavage (immunostaining and NH₂-terminal amino acid sequence). (B) Comparison of the NH₂-terminal amino acids of the p15 fragment with human MnSOD. The arrows point to the location of V8 protease cleavage and mark the p15-fragment; one letter code was used. These sequence data are available from EMBL/GenBank/DBJ under accession number Y00472.

antibodies reached their maximum value with titers up to 1:3,200 at the onset of symptoms of IM, and fell below the detection limit within 6–8 wk. Patients' recovery coincided with the fall of autoantibodies.

Discussion

In the course of IM, patients are producing a number of autoantibodies. Hitherto, only antibodies against the blood

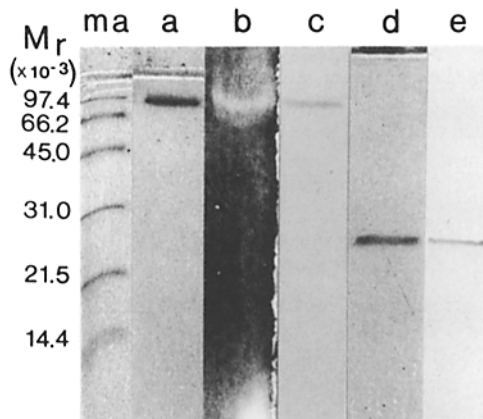


Figure 3. Demonstration of the serological identity of p26 and MnSOD. Lanes a, b, and c, MnSOD from human liver after native PAGE (lane a, silver staining; lane b, demonstration of the enzyme activity; lane c, reaction with affinity purified IgM anti-p26 antibodies); lanes d and e, MnSOD from human liver tissue after denaturing SDS-PAGE (lane d, silver staining; lane e, reaction with affinity purified IgM anti-p26 antibodies).

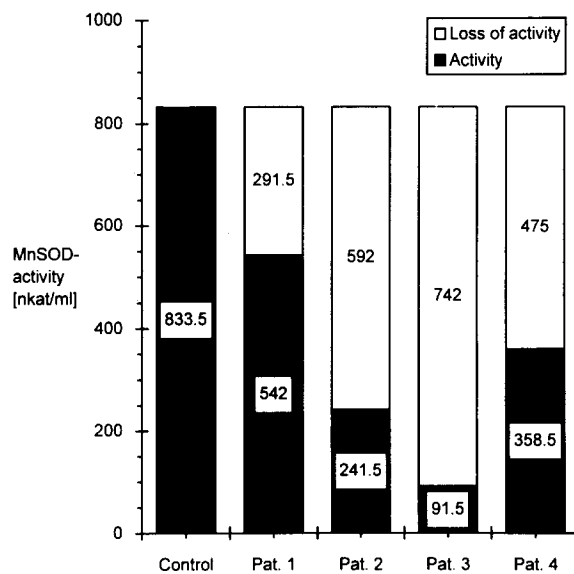


Figure 4. Inhibition of human MnSOD by affinity purified antibodies from sera of patients with acute EBV infection.

group antigen "i" (20) and antibodies against TPI are known to be clinically significant (3). Both antibodies are responsible for hemolysis and hemolytic anemia, which is a serious complication of IM. We assume that the detection of autoantibodies against p26 and the presented identification of p26 as MnSOD may contribute to the understanding of the immunopathogenesis of IM.

MnSOD catalyzes the dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen. Besides the intracellular presence of MnSOD, the enzyme is found in human serum in high concentrations (21). It protects capillary endothelial cells against the damaging effects of superoxide (22). The autoantibodies we detected in sera of patients with acute EBV infection were capable of blocking the function of MnSOD. We suspect that this harmful mechanism causes the common physical signs in IM patients, such as disturbed liver function, gelatinous appearance of the soft palate, supra-orbital edema, and exanthema. The well-known biochemical and histopathological changes in the acute phase of EBV infections may be quoted to sustain our hypothesis; most of the lytically or latently infected B cells perish due to the virus production or the immune reaction, which mainly takes place in the perivascular space, especially in lymphatic organs. Due to the destruction of virus-infected lymphocytes, the high turnover of nucleotides leads to increased activity of the xanthine oxidase, as was measured by an increased level of uric acid in sera of IM patients. The enhanced catabolism of purine bases by the xanthine oxidase leads to an excessive production of oxygen radicals (23). If MnSOD is blocked by the autoantibody, the removal of oxygen radicals is inhibited. A rise of the oxygen radical concentration has been observed to lead to a physiological cascade resulting in leukocyte margination and vascular leakage (24). In transgenic mice evidence was given that oxygen injury increased the permeability of capillary endothelium, whereas MnSOD had a protective influence (22). Antibodies were able to suspend the action

of MnSOD. Recent work demonstrated that blocking the SOD activity in the membrane of *Nocardia asteroides* by monoclonal antibodies rendered the bacterium highly susceptible to the toxic superoxide radicals (25). These observations are important in regard to our conception.

High autoantibody titers against MnSOD were detectable in all patients with acute EBV infections, but not in patients with other acute herpesvirus infections or in healthy probands. Only one patient with acute cytomegalovirus infection showed a weak reaction to MnSOD. In vitro the inhibi-

tory activity of the purified autoantibodies against MnSOD varied among individual patients. According to our data, a strong inhibitory activity may be connected with a severe clinical course of IM. Sufficient amounts of serum for affinity purification of anti-MnSOD antibodies necessary for the performance of the inhibiting assay, as well as detailed clinical data were, however, only available in few patients. Thus, a correlation between the activity of the anti-MnSOD antibodies and the severity of the disease will have to be left to a future prospective study.

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