Brief Definitive Report

AUTOANTIBODIES AGAINST TRIOSEPHOSPHATE ISOMERASE

A Possible Clue to Pathogenesis of Hemolytic Anemia in Infectious Mononucleosis

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Various clinical and pathological conditions can develop after EBV infections depending on the age of the individual. Whereas young children show a silent seroconversion, adolescents with acute EBV infection develop infectious mononucleosis (IM) in half of the cases. Being a lymphotropic virus, EBV can trigger an unspecific B cell stimulation besides the specific immune response. This may cause a big rise of class G and M Igs in the sera. Little is known about their specificity. Heterophil antibodies appear that agglutinate sheep, horse, and bovine erythrocytes (1, 2). Cold antibody with anti-i specificity was found in 8% to 60–70% of the IM patients (3, 4). In addition, antibodies are found that react with components of the cytoskeleton (5) or possess antinuclear features (6). None of these antibodies always appears in acute EBV infection, thus, their significance for the pathogenesis of IM is not clear. This may be different in the antibodies we recently detected in all sera of persons acutely infected with EBV. These antibodies possess specificity for the glycolytic enzyme, triosephosphate isomerase (TPI).

Materials and Methods

Preparing the Antigens.   Frozen cells (1 ml packed volume) and disrupted tissues were allowed to thaw, and were subsequently resuspended in equal volumes of modified lysis buffer (7) containing 9.5 M urea, 2% (wt/vol) NP-40, and 5% β-ME; further extraction was done by five cycles of freezing and thawing (−20°C/room temperature). The cell lysate was then centrifuged at 3,000 g for 15 min. The supernatant fluid was used for antigen preparation by SDS-PAGE followed by immunoblotting. The protein concentration (20 mg/ml) was measured by the method of Neuhoff et al. (8).

PAGE.   Composition and electrophoresis of SDS-13% (wt/vol) polyacrylamide slab gels were performed as reported by Laemmli (9). The electrotransfer of proteins from SDS gels to hydrophobe polyvinylilenedifluoride (PVDF) membrane (Millipore Continental Water Systems, Bedford, MA) was performed by transversal electrophoresis (10).

Immunostaining the Blots.   For the detection of antibodies, sera, as well as secondary antibodies conjugated with peroxidase, were diluted in 20% FCS/PBS (vol/vol). Each antibody

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was incubated while continuously shaken for 1 h at room temperature. The sera were diluted 1:50, as the second antibody, a peroxidase conjugated anti–human IgM, was used in a dilution of 1:1,000.

Isolation and Purification of p26/p29.   P26 and p29 were isolated from Raji cell lysate by means of electroelution (11) after preparative SDS-13% PAGE. After further purification on an SDS-10-18% gradient polyacrylamide gel, the electrotransfer on PVDF for the protein sequencing was performed.

Cyanogen Bromide Cleavage.   Purified p29 was cleaved by cyanogen bromide according to the method of Schroeder et al. (12). The fragments were separated in SDS-18% PAGE.

Determination of Amino Acid Sequence.   For the determination of amino acid sequence, an automatic gas phase sequencer (470 A; Applied Biosystems; Inc., Foster City, CA) was used (13).

TPI from other Species.   Commercially available TPI from dog, pig, and rabbit (muscle tissue), as well as from yeast (Sigma Chemical Co., FRG) were used for comparison. For gel electrophoresis, protein concentrations were adjusted to 1 mg/ml.

Affinity Purification of Anti-TPI Antibodies.   Electrophoretic transfer of p29 (human TPI) from gels to PVDF membrane was carried out as described elsewhere (10). After transfer, the PVDF membrane was treated with sera from IM patients. The elution of antibodies was performed using the method described by Olmsted (14).

^{51}Cr *Release Assay.*   500 μl of a 10% suspension of human erythrocytes group 0 Rhesus negative was marked with 5 μCi ^{51}Cr for 1 h at room temperature. 10 μl of ^{51}Cr-marked erythrocytes was incubated with 10 μl of inactivated proband's serum for 2 h at 37°C. Afterwards, 10 μl of guinea pig serum was added as a source for complement. Guinea pig serum had previously been absorbed on human erythrocytes blood group O, Rhesus negative for 4 h at room temperature. Each assay was done in quadruplicate. Percentage of ^{51}Cr release was calculated as: 100× (cpm in supernatant/cpm total).

Results and Discussion

When investigating the IgM fractions of IM sera, it was striking that all IM sera cause a cytoplasmic fluorescence not only in EBV genome-positive cells, but also in negative cells. This reaction was missing with EBV seronegative sera. For identification of the antigens (see Fig. 1), EBV genome-positive and -negative cells
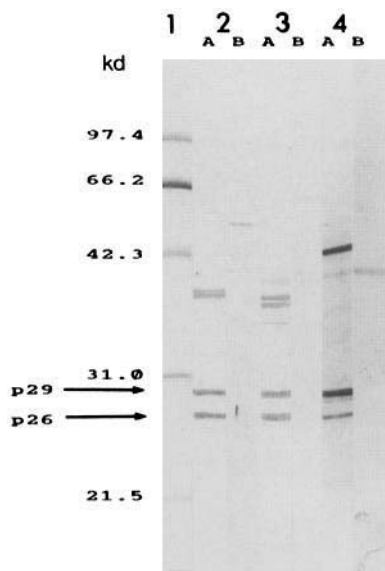

FIGURE 1.   Detection of p26/p29 in cell lysates. (Lane *1*) Protein marker coomassie blue stained; (lane *2*) Raji lysate; (lane *3*) MOLT 4 cell lysate; (lane *4*) human erythrocyte group 0 lysate. The reaction of the cell lysates was performed with serum deriving from a patient in the acute stage of IM (*A*) and with serum from a healthy volunteer (*B*).

were lysed, and the lysate was separated in the SDS-PAGE. After electrotransfer on a PVDF membrane, they were used for the immune reaction with IM sera. All IM sera reacted with two cellular structural components that were identified as proteins of a molecular mass of 26,000 (p26) and 29,000 daltons (p29), respectively. Both proteins are present in all mammalian cells investigated: human lymphoblastoid cell lines, primary human lymphocytes, and human erythrocytes. They were also found in mouse cells, as well as in rabbit and duck erythrocytes.

The antibodies against p26 and p29 found in all 182 IM sera tested all belonged to the Ig class M (IgM). They were not detected before EBV infection. IgM anti-p26 and IgM anti-p29 appear in high levels in sera of IM patients at the time of the outbreak of clinical symptoms. Their rise and fall is very similar to that of the IgM antibodies against the EBV capsid antigen. 5–6 mo later, anti-p26/anti-p29 are no longer detectable. A switch to IgG does not occur.

Both regularity and intensity of the immunological reaction were reasons for further work and identification of the cellular antigens p26 and p29. The two proteins were isolated from Raji cell lysates. After purification the sequence of the 11 NH_2-terminal amino acids of p29 was performed with a gasphase sequencer. The sequence gained was compared with the data of the Dayhoff chart. Apart from an exchange at position 5_{Pro} vs. 5_{Lys}, the sequence of the amino acids coincided with that of human TPI. In addition, the sequences of the fragments gained by cyanogen bromide cleavage were in concordance with TPI (see Fig. 2). TPI consists of two identical parts with a molecular mass of 26,500 each. P26 is blocked NH_2 terminally. It will be analyzed soon.

To find out whether IgM anti-p29 really recognizes TPI, commercially available TPI preparations were treated with IM sera (see Fig. 3). Whereas IgM anti-p29 recognized human, rabbit, and dog TPI, there was no reaction with TPI derived from pig and yeast. These data correspond with the sequence data stating an ho-
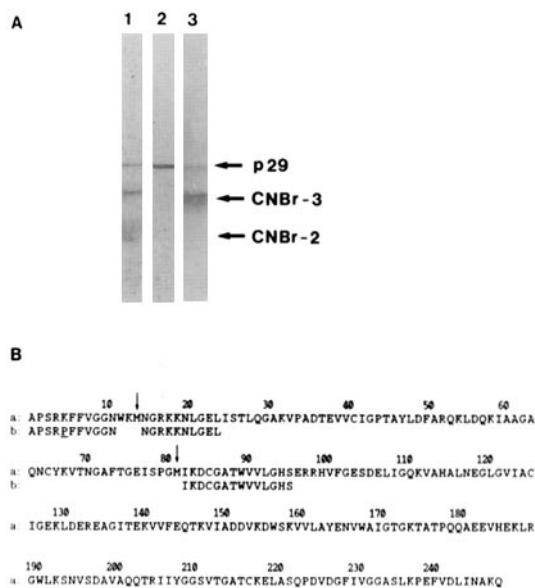

FIGURE 2.   Identification of p29. (A) Lane 1, p29 after CNBr-cleavage (coomassie blue); lane 2, purified p29 (coomassie blue); lane 3, p29 after CNBr-cleavage (immunoblot). (B) a, Amino acid sequence of human TPI (15); b, p29, 11 amino acids from NH_2 terminus, 10 amino acids from the CNBr-fragment 2, 14 amino acids from the CNBr-fragment 3. The arrows point to the location of CNBr cleavage; underlining shows exchange of amino acids; one letter code was used.
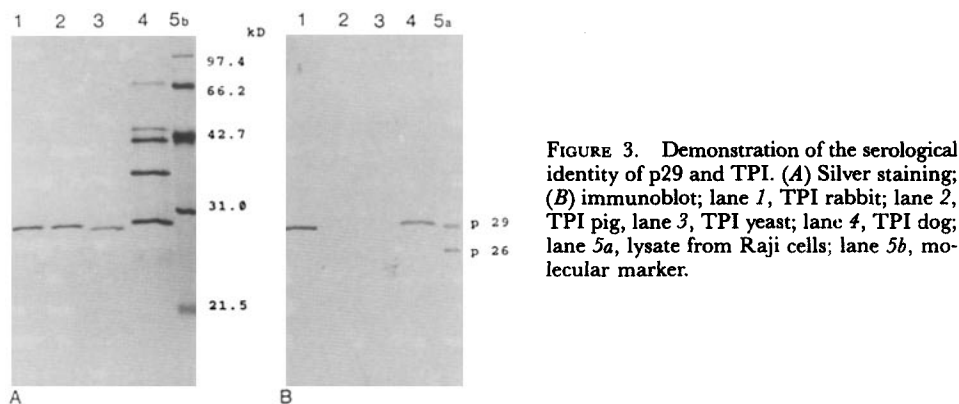
FIGURE 3. Demonstration of the serological identity of p29 and TPI. (*A*) Silver staining; (*B*) immunoblot; lane *1*, TPI rabbit; lane *2*, TPI pig, lane *3*, TPI yeast; lane *4*, TPI dog; lane *5a*, lysate from Raji cells; lane *5b*, molecular marker.

mology of >90% between man and rabbit (15), but only of 40% between man and yeast (16). The amino acid sequences of TPI from dogs and pigs are not known (Dayhoff protein data base). Further, it was demonstrated that rabbit TPI absorbed anti-p29 antibodies from IM sera (data not shown). Thus, p29 is one of the two subunits of TPI. The IgM anti-p29 antibodies produced in the course of acute EBV infection are directed against TPI.

TPI is a glycolysis enzyme. Cells that take their energy mainly from glycolysis are particularly susceptible to disturbances of the enzyme function leading to membrane instabilities, spherocytosis, and in most serious cases, to hemolysis (17). The full picture of hemolytic anemia is a rare complication of IM, but often mild and multifactorial anemia is found in the disease. Several reports have linked the anti-i antibody with hemolytic phenomena in IM (18, 19). The sera taken from IM patients suffering from hemolytic anemia, however, proved to be negative for anti-i antibodies (20). It seems likely that additional mechanisms are effective in the cases of hemolytic anemia accompanying IM.

Antibodies against TPI taken from IM sera purified in their affinity bind to O, Rh⁻ RBC, and induce a ^{51}Cr release in the presence of complement. An antibody against p29 induced in rabbits is distinctively effective (see Fig. 4).
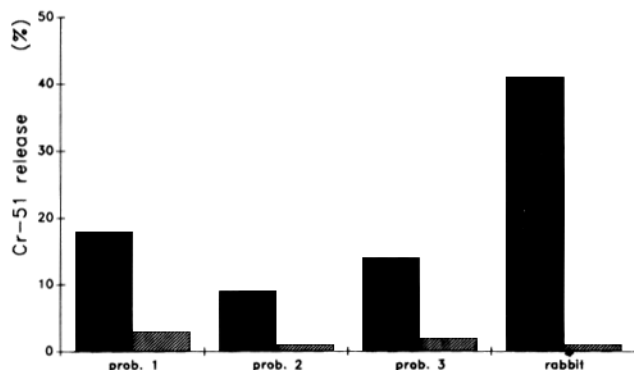

FIGURE 4. ^{51}Cr release from human erythrocytes group O, Rhesus negative. Reaction of serum negative for IgM anti-p29 (*hatched bars*); reaction of serum positive for IgM anti-p29 (*solid bars*).

We assume that the anti-TPI antibody is responsible for disturbances of erythrocytes in patients with IM; however, as yet, we have no firm evidence concerning the mechanism. The ^{51}Cr release in vitro is not accompanied by massive lysis of the RBC. Also, we would not expect this according to the in vivo situation, as all patients with IM produce anti-TPI, but only 3% develop hemolysis. It is conceivable that the effect of anti-TPI is a two-step mechanism: first, an initial reaction on the membrane requiring complement, which is then followed by an influx of anti-TPI into the cell where it acts. The extent of the initial damage of the membrane would influence the further incidence of hemolysis. The reaction on the membrane may be understood as a molecular mimicry between TPI and a component of the outer section of the erythrocyte membrane. Further information concerning the epitope of TPI that is recognized by anti-TIM of IM patients will be of interest.

Summary

In sera from patients with acute EBV, infection and the clinical symptoms of infectious mononucleosis antibodies of the Ig class M were found that are directed against two cellular proteins. The molecular mass of these proteins was determined to be 29 (p29) and 26 kD (p26), respectively, in SDS-PAGE. P29 was identified as part of the glycolytic enzyme triosephosphate isomerase (TPI) by comparison of the NH_2-terminal amino acid sequences. A purified antibody against TPI induces a ^{51}Cr release from human erythrocytes. Possibly, anti-TPI causes hemolysis, which is an infrequent but serious symptom of infectious mononucleosis.

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