We observed the presence of a new autoantibody, anti-erythrocyte protein 4.1, in a patient with autoimmune hemolytic anemia (AIHA). Western blotting analysis revealed that IgG from the patient’s plasma reacted with erythrocyte protein 4.1. However, among other patients with hemolytic diseases (six having AIHA and three each having either hereditary spherocytosis, elliptocytosis, or lead poisoning) as well as among control subjects, no antibody activity to protein 4.1 was observed. In addition to the anti–protein 4.1 antibody, two different kinds of anti-erythrocyte antibodies were detected by conventional serological studies in this patient. One of them was an anti-EN-like antibody in the eluate from the patient’s erythrocyte, while another was the anti-S-specific antibody in the plasma. An elution study and an absorption study using S antigen–positive erythrocytes demonstrated that the anti–protein 4.1 antibody differed from both the anti–EN-like antibody and the anti-S antibody. Familial analysis of the patient revealed the same antibody in her brother, who did not have hemolytic anemia. These results demonstrate that anti–protein 4.1 antibody is considered to be included in the spectrum of anticytoskeleton autoantibodies, which have been observed in patients having increased cell lysis as well as in healthy subjects.

**Case Report**

A 61-year-old woman was admitted for the evaluation of slowly progressive anemia and jaundice in August 1981. She had delivered four children, never received a blood transfusion, and had no episode of hemoglobinuria. Physical examination showed mild splenomegaly. Peripheral blood analysis revealed that the hemoglobin concentration was 8.0 g/dL, the hematocrit value was 22.4%, and the RBC count was 190 x 10^6/μL with 12.1% reticulocytes. Neither elliptocytosis nor spherocytosis was present. A bone marrow aspiration revealed erythroid hyperplasia. Blood chemistry showed an elevated serum indirect bilirubin concentration of 1.7 mg/dL and an elevated serum lactate dehydrogenase level of 661 U/L. Both direct and indirect antiglobulin test results were positive, but tests for antinuclear antibody as well as other autoantibodies were all negative. A diagnosis of AIHA was made, and treatment with prednisolone (15 mg/d) was started. The anemia recovered promptly, but a relapse was observed after decreasing the dose of prednisolone during outpatient treatment. There was no change in the antiglobulin test results during the treatment period.

Other patients with hemolytic diseases (six with AIHA, and three each having either HS, elliptocytosis, or lead poisoning), some control subjects (including ten age-matched subjects), as well as the patient’s brother and daughter were also examined in this study. There was no evidence of hemolysis in either the patient’s brother or daughter at observation.

**Materials and Methods**

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**Methods**

**Serological studies.** The ABO, Rh, Lewis, MN, and P systems of the patient’s erythrocytes were analyzed by regular blood banking methods. To identify other systems of the blood group, the patient’s erythrocytes were treated with chloroquine diphasphate for up to two hours. The direct antiglobulin test (DAT) was performed by using polyspecific antihuman serum (AHS); nonspecific antihuman IgG, IgA, and IgM; and antihuman complements (Ortho Pharmaceutical Corp, Raritan NJ). Anti-IgG1, anti-IgG2, anti-IgG3, and anti-IgG4 subclass–specific antisera (Janssen Pharmaceuticaals, Beerse, Belgium) were also used. The blood group specificity of antibodies, both in the eluate and serum, was checked by the Resolve panel (Ortho) with the indirect antiglobulin technique at 37°C. The eluate was obtained from the patient’s erythrocytes by using a dichloromethane-trichlorotrifluoroethane solution (Ortho, Tokyo). Antibody reactivities with Rh null and En(a-) erythrocytes were also examined.

**Absorption study.** Washed and packed erythrocytes possessing the S antigen were incubated with an equal volume of plasma from the patient at 37°C for 60 minutes. After centrifugation at 3,000 rpm for five minutes, erythrocytes and absorbed plasma were obtained. These steps were repeated twice. After each absorption, the eluates from treated erythrocytes and absorbed plasma were tested to confirm the efficiency of absorption.

**Preparation of erythrocyte ghosts and Triton X-100 fractions.** Anticoagulated blood samples taken from all of our subjects were centrifuged at 3,000 rpm for 5 minutes, and the resulting erythrocyte pellets were washed and then resuspended in phosphate-buffered saline (pH 7.4) with 5% dimethyl sulfoxide. The cells were then lysed with 0.5% Triton X-100 and centrifuged again. The resulting pellets were resuspended in buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) and stored at -80°C. The membranes were then extracted with chloroform-methanol (2:1, v/v) and then assayed by Western blotting.

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were centrifuged at 3,000 rpm for ten minutes to separate the erythrocytes and plasma. The erythrocytes were washed three times with physiological saline. They were then lysed in 10 mmol/L Tris-HCl, pH 7.4, and centrifuged at 17,000 rpm for 15 minutes at 4°C. The pellet was washed five times with the lysing buffer. Erythrocyte ghosts from a healthy subject were stirred with an equal volume of 2% Triton X-100, 0.3 mol/L NaCl, and 20 mmol/L Tris-HCl, pH 7.4, for 30 minutes at 4°C and centrifuged at 17,000 rpm for 15 minutes at 4°C. The pellet was washed three times with the same buffer.

**Purification of protein 4.1.** Protein 4.1 was purified from outdated bank blood by the method of Becker et al. Briefly, glyceraldehyde-3-phosphate dehydrogenase-, spectrin-, and actin-depleted ghosts were treated with a buffer containing 2% Tween 20. The supernatant of this extract was chromatographed on a diethyl aminomethyl–cellulose column, and the protein of the major peak was collected.

*Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.* Erythrocyte ghosts, Triton X-100 fractions, and purified protein 4.1 were analyzed on sodium dodecyl sulfate (SDS)–polyacrylamide gels by using the discontinuous system of Laemmli and stained with Coomassie blue and Stains-all (Bio-Rad, Richmond, CA). The nomenclature for the erythrocyte membrane proteins was adopted according to the system proposed by Fairbanks et al and modified by Steck.

**Western blotting.** Western blotting was performed by using the method of Towbin et al. Proteins separated on SDS–polyacrylamide gels were electrophoretically transferred to the filter papers. After protein-free sites on the filter papers were blocked with a buffer containing 3% gelatin, the filter papers were incubated with either the plasma of the subjects at a dilution of 1:10 or with the eluate from erythrocytes for two hours at room temperature. After washing, the filter papers were incubated with peroxidase-conjugated antihuman IgG (Cappel Laboratories, Malvern, PA) at a dilution of 1:250 for one hour at room temperature. After washing, the filter papers were incubated with peroxidase-conjugated antihuman IgG (Cappel Laboratories, Malvern, PA) at a dilution of 1:250 for one hour at room temperature. After washing, color development was performed by using diaminobenzidine and hydrogen peroxide.

**RESULTS**

**Serological Studies**

Blood groups of the patient’s erythrocytes were as follows: A; D + C + E – c – e +; Le(a + b –); M-N +; P1 +. The patient’s Ss phenotype could not be identified because of a positive DAT response after chloroquine treatment. Agglutination scores of the DAT with AHS, anti-IgG, anti-IgA, anti-IgM, anti-C3d, and anti-~C3b–C3d~C4 were 3 +, 3 +, −, −, −, −, and −, respectively. Serological analysis using the Resolve panel showed that this patient had two different kinds of antierthrocyte antibodies. One of them was a so-called pan-reacting antibody in the eluate from the patient’s erythrocytes, whereas another was the anti-S-specific antibody in the serum (Table 1). The IgG subclasses of these antibodies were different; the former was IgG1, and the latter was IgG1 plus IgG3. To further analyze the antigenic specificity of the autoantibody in the eluate, tests for reactivities with Rh null and En(a –) erythrocytes were performed. As a control R1R1 erythrocytes were used. Titters against R1R1, Rh null, and En(a –) erythrocytes were 512, 512, and 4, respectively. Although the autoantibody in the eluate had anti-En* specificity, there was a possibility of the antibody being anti-Wr*. Since this antibody was not tested against En(a +), Wr(a + b –) erythrocytes, it was regarded as an anti-En*–like antibody.

**Identification of the Autoantibody Against Protein 4.1**

SDS–polyacrylamide gel stained with Coomassie blue revealed no abnormal findings among erythrocyte membrane proteins in this patient (Fig 1A). However, Western blotting analysis demonstrated that erythrocyte ghosts from this patient contained a large amount of IgG-related 54-Kd protein (Fig 1B, lane a). A small amount of this protein was also detected in another AIHA patient (Fig 1B, lane b). Since erythrocyte ghosts were treated with a buffer containing a reducing agent, erythrocyte membrane–bound IgG molecules were naturally considered to be reduced to both heavy chains and light chains. Thus, it was concluded that the 54-Kd protein was identical with the IgG heavy chain based on its mol wt and its immunoreactivity with anti-IgG. Quantitative differences between the amount of IgG derived from the patient’s erythrocyte ghosts and that of another AIHA patient were proportional to agglutination scores in the DAT. Western blotting probed with the patient’s plasma and with peroxidase-conjugated antihuman IgG showed that the patient’s IgG reacted with a 78-Kd protein of erythrocyte ghosts from the patient as well as from other subjects (Fig 1C). To further analyze the 78-Kd protein, erythrocyte ghosts were separated into two fractions by selective solubilization using Triton X-100. The patient’s IgG reacted only with the 78-Kd protein that existed in the Triton X-100–insoluble fraction (data not shown). Since the 78-Kd protein was likely to be protein 4.1, the reactivity of the patient’s IgG with purified protein 4.1 was examined. It was demonstrated that this patient had the anti–protein 4.1 antibody (Fig 2B, lane a). Considering the other subjects, the patient’s brother had the same antibody (Fig 2B, lane b), but the others did not.

**Elution and Absorption Studies**

Both elution and absorption studies were performed to clarify the relationship between various antierthrocyte antibodies detected by the antiglobulin test and the autoantibody against protein 4.1. IgG in the eluate from the patient’s
DISCUSSION

The present study demonstrates that one patient with AIHA had three different kinds of antibodies against erythrocyte components: anti-En<sup>a</sup>-like as well as anti-S-specific antibodies detected by serological studies and the anti-protein 4.1 antibody detected by Western blotting. An elution study and an absorption study using S antigen-positive erythrocytes showed that the anti-protein 4.1 antibody differed from both the anti-En<sup>a</sup>-like antibody and the anti-S antibody. Other patients with hemolytic diseases (six with AIHA and three each having either HS, elliptocytosis, or lead poisoning) as well as some control subjects had no antibody against protein 4.1.

Anti-protein 4.1 autoantibody is considered to be included in the spectrum of anticytoskeleton autoantibodies. Several
anticytoskeleton antibodies have been reported in both healthy subjects and in patients having increased cell lysis.\textsuperscript{20-23} It is suggested that these antibodies may have a physiological role in the clearance of debris from lysed cells.\textsuperscript{23} Although Lutz and Wipf\textsuperscript{25} have demonstrated that there are naturally occurring autoantibodies to cytoskeletal proteins from human erythrocytes, the autoantibody against protein 4.1 has not been described. Thus, the present cases (the patient with AIHA and her brother) appear to be unique in that they have a circulating antibody against protein 4.1.

There are several possibilities whereby anti–protein 4.1 antibody could have arisen in our patient and her brother. First, they may have had a primary abnormality of protein 4.1 (unusually stable protein 4.1) or an abnormal metabolism of protein 4.1 (some special enzyme deficiencies to cleave protein 4.1). Protein 4.1 is an unstable protein\textsuperscript{29} and is easily cleaved by chymotrypsin and calpain.\textsuperscript{24,25} Protein 4.1 released from erythrocytes is also usually thought of as not having immunogenicity in vivo. If they have such abnormalities in protein 4.1, then protein 4.1 or its degrade may stimulate the antibody production after the destruction of erythrocytes. Further analysis of their own erythrocyte protein 4.1 or special enzymes is needed to solve this problem. Second, anti–protein 4.1 antibody may be an age-related autoantibody. Our patient and her brother are in advanced age. It has been shown that autoantibodies to nucleic acids, smooth muscle, mitochondria, lymphocytes, gastric parietal cells, immunoglobulin, and thyroglobulin are found with increased frequency in elderly humans.\textsuperscript{26} In our study, we examined ten age-matched control subjects who did not have hematologic or immunologic disorders and found that there was no anti–protein 4.1 antibody in any of them. A more extended survey using perhaps several hundred elderly people will be needed to clarify this possibility. Finally, anti–protein 4.1 antibody may be associated with other factors. Autoantibodies with specificity for erythrocyte antigens have been described in association with a number of underlying conditions, for example, lymphoproliferative disorders, rheumatic disorders, certain infections, certain nonlymphoid neoplasms, certain chronic inflammatory diseases, and the ingestion of certain drugs.\textsuperscript{27} However, neither our patient nor her brother had such recognizable conditions.

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Autoantibody against erythrocyte protein 4.1 in a patient with autoimmune hemolytic anemia

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