Autoimmune haemolytic anaemia is associated with a defect in Fas-mediated apoptosis: a case report

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Self-reactive T cells are deleted from the thymus by a negative selection mechanism during early T-cell differentiation.[1–3] However, self-reactive mature T cells cannot be deleted in this way as their elimination requires Fas-mediated apoptosis.[4] Fas is constitutively expressed in many cells, including T cells,[5] and its ligation by FasL results in the activation of the Fas-associated death domain (FADD) and caspases.[6–8] Autoimmunity results when the activation-induced cell death (AICD)-resistant T cells accumulate in the periphery of the gland. Homozygous Fas mutations are rare,[9,10] however, Dianzani *et al*. demonstrated that autoimmunity and lymphoproliferation also develop in the absence of Fas mutation.[11]

Haemolytic anaemia encompasses a heterogeneous group of intrinsic red blood cell (RBC) defects in which either the structural or functional integrity is damaged, resulting in a reduction in RBC survival. However, the molecular defect underlying the haematologically important erythroenzyme disorders has been elucidated.[12–14]

This case study aims to investigate the presence of defective Fas-mediated apoptosis in a 55-year-old Saudi female patient with autoimmune haemolytic anaemia (AIHA).

At diagnosis, the patient was anaemic with thrombocytosis. She was lymphopenic and had a high neutrophil count. Laboratory results at presentation are summarised in Table 1. After obtaining appropriate consent, peripheral blood was obtained and peripheral blood mononuclear cells (PBMNCs) were isolated over a Ficoll-Hypaque density gradient (Nycomed Pharma, Oslo, Norway).

$CD34^+$ cells were separated using MiniMacs magnetic immunoaffinity columns (Milteny Biotec), following the manufacturer's instructions. Briefly, cells from pooled granulocyte-macrophage colony-forming units (GM-CFU) were incubated for 15 min at 4°C with 100 µL blocking FcR (A1 reagent), 100 µL hapten-conjugated anti-$CD34^+$ monoclonal antibody (A2 reagent) and 300 µL MiniMacs (MM) buffer containing calcium- and magnesium-free phosphate-buffered saline (PBS; GibcoBRL), 0.5% human serum albumin (ImmunoAG) and 5 mmol/L EDTA (GibcoBRL). Following a single wash in 10 mL MM buffer, cells were resuspended in 100 µL anti-hapten monoclonal antibody conjugated to beads (reagent B) and 400 µL MM buffer, and then incubated at 4°C for 15 min. The cells were washed again in 10 mL MM buffer and gently resuspended in 1 mL MM buffer. The cell sample was loaded on to the affinity column held in a magnet and washed through with 4 x 0.5 mL MM buffer. After removal from the magnet, $CD34^+$ cells were expelled from the column with 1 mL MM buffer.

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Table 1. Laboratory results at presentation.

Test	Value
White blood cells	24.3 x 10^9/L
Red blood cells	3.2 x 10^{12}/L
Haemoglobin	085 g/L
Platelets	838 x 10^9/L
Lymphocytes	11%
Neutrophils	78%
Monocytes	6%

To confirm defective Fas-mediated lymphocyte apoptosis, cells were cultured with phytohaemaglutinin (PHA; 10 µg/mL) for 2 days, washed and then incubated with 10 µg/mL isotype-control mouse IgM or anti-Fas monoclonal IgM CH-11 (Upstate Biotechnology, NY, USA) for 24 h. The percentage of apoptotic cells was determined by an apoptosis detection assay (TUNEL; Oncogene, MA, USA).

Fas expression on lymphocytes was determined by staining PBMNC with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-Fas antibody (Becton Dickinson, MA, USA) and analysed using a FACScan flow cytometer running CellQUEST software. Appropriate negative controls were used, and experiments were performed in replicate.

To identify Fas mutation in the presence of a defect in lymphocyte apoptosis, specific primers were used to amplify Fas complementary DNA (cDNA) derived from PBMNCs. FasR: 5′-TACAGCCAGCTATTAAGAATCT-3′ (151–172 bp) and FasF: 5′-GGACATGGCTTAGAAGTGGA-3′ (845–826 bp). The primers were designed by Dr A. Chandraskekran (Department of Haematology, ICSM, London) using the Pride programme. After polymerase chain reaction (PCR) amplification, cDNA was subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA, USA). Ten clones were sequenced by an automated fluorescence sequencer.

Fig. 1. Fas functional assay on activated lymphocytes was impaired on the patient. Lymphocytes were treated with the apoptosis-inducing monoclonal anti-Fas CH-11 or isotype control. The percentage of apoptotic cells was measured by TUNEL assay.

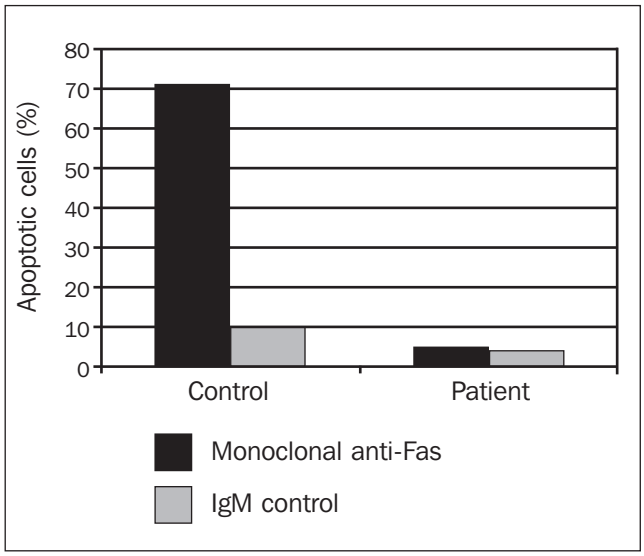
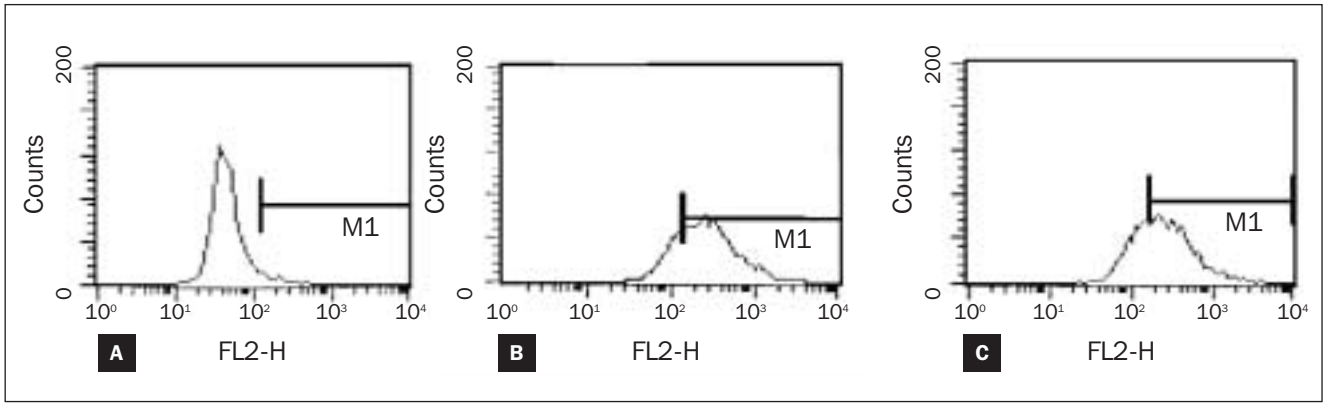


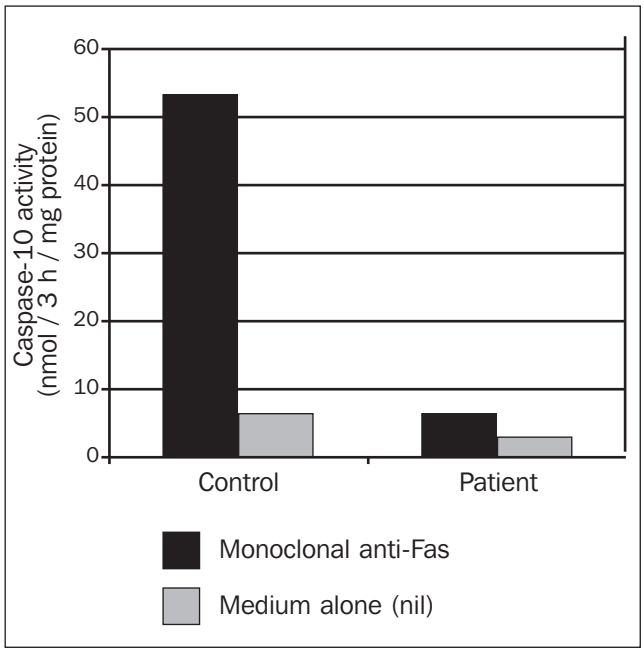
Fig. 2. Normal Fas expression on T lymphocytes. One representative experiment out of four with similar results using (a) the isotype control, (b) the controls and (c) the patient's cells.



Caspase 10 activity was measured using a fluorimetric substrate cleavage method. Briefly, the patient's T lymphocytes were treated with 10 µg/mL anti-Fas CH-11 monoclonal antibody for 24 h, harvested and washed once in PBS. Cell pellets were lysed in 20 mmol/L Tris, 137 mmol/L NaCl, 1% NP40 detergent and 10% glycerol. Protein content was determined by Bradford assay. Activity was measured as fluorescence emission following the addition of an appropriate fluorogenic peptide substrate (aminomethylcoumarin [AMC] derivative; R&D, Oxford, UK) and incubated at 37°C for 3 h. Release of free AMC was monitored fluorometrically at an emission wavelength of 460 nm. Experiments were performed in replicate.

In order to determine whether or not autoimmunity was secondary to alterations in the pattern of lymphocyte apoptosis, these cells were incubated with monoclonal anti-Fas CH-11. However, the patient's lymphocytes showed no increase in apoptosis in the presence of anti-Fas CH-11. Fas specificity in this assay was determined using Jurkat cells (Fas^+ cells) as the control (Fig. 1).

Fig. 3. Caspase-10 activity.
One representative experiment of three shown.



In order to determine whether or not the defect in Fas signalling was related to a decrease in Fas expression on the lymphocytes, flow cytometry was performed using FITC-conjugated anti-Fas as the marker. In this patient, Fas expression on lymphocytes was comparable to that on the normal control cells. Thus, the data suggest that a defect in Fas expression was not responsible for the altered Fas signalling (Fig. 2).

Having established that the patient's lymphocytes were resistant to Fas-induced apoptosis, the study then focused on whether or not a mutation was responsible for the defect in Fas signalling. The Fas receptor was sequenced and a gene mutation was found at cDNA nucleotide 960 (C→A substitution).

Next, the possible involvement of caspases in the reduction of apoptosis in AIHA was investigated, in order to discover whether or not a defect in caspase-10 activation is responsible. To clarify the mechanism of caspase-10 impairment, enzyme activity was assessed in a cell lysate using fluorimetric analysis. Monoclonal anti-Fas CH-11 treatment induced caspase-10 activity in the control cells but activity was impaired in the patient's T lymphocytes. (Fig. 3).

In this study, the presence of a defect in lymphocyte apoptosis was found to be associated with AIHA. The elimination of autoreactive lymphocytes was inhibited in the presence of a Fas mutation, either due to a decrease in surface expression or to the presence of inhibitory proteins (e.g., FLICE-inhibitory proteins [FLIP]).[15,16] The defects are similar to those seen in autoimmune lymphoproliferative syndrome (ALPS), which is linked to a genetic mutation in Fas or to other Fas pathway molecules such as FasL and caspase-10.[9]

In the patient described, lymphocyte apoptosis was not induced in T lymphocytes via Fas/FasL; however, other candidate pathways may include tumour necrosis factor receptor-1 (TNFR-1) and TNF receptor-associated inducing ligand (TRAIL) but their physiological importance remains unclear, even in mouse models. Thus, the presence of the Fas mutation is likely to be the single cause for the patient's AIHA, as is the case in patients with ALPS.[9,17] However, it remains unclear whether or not the defect in lymphocyte apoptosis preceded the clinical events (i.e., lymphadenopathy and an increase in $CD3^+$ $CD4^-$ $CD8^-$ T cells in the peripheral blood).

The results presented here are consistent with the work of Wang and colleagues,[18] who showed that caspase-10

deficiency in humans is linked to defective lymphocyte apoptosis, which suggests that AIHA may require caspase function for the induction of death receptor-mediated apoptosis. However, altered Fas pathway signalling may contribute to a new aetiology for AIHA. □

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