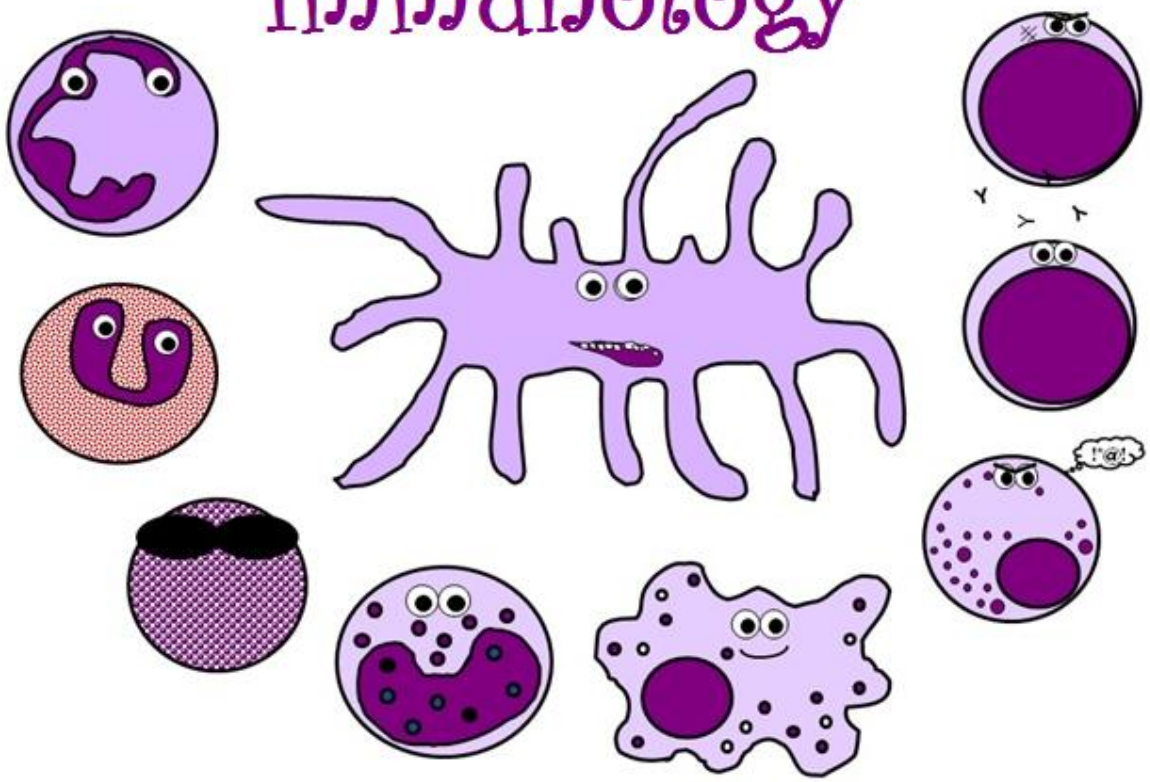
Immunology



Sheet Slides

Number: 13

Subject: ALPS

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27/11

Shelling

CASE 19 Autoimmune Lymphoproliferative Syndrome (ALPS)

Increased survival of lymphocytes as a result of a mutation in Fas.

When antigen-specific lymphocytes are activated through their antigen receptors, they undergo blast transformation and then begin to increase their numbers exponentially by cell division. This clonal expansion can continue for up to 7 or 8 days, so that lymphocytes specific for the infecting antigen increase vastly in number and can come to predominate in the population. In the response to certain viruses, 50% or more of the CD8 T cells at the peak of the response are specific for a single virus-derived peptide:MHC class I complex. After clonal expansion, the activated lymphocytes undergo their final differentiation into effector cells; these remove the pathogen from the body and so terminate the antigenic stimulus.

When an infection has been overcome, activated effector T cells are no longer needed, and cessation of the antigenic stimulus prompts them to undergo programmed cell death, or apoptosis (Fig. 19.1). Apoptosis is widespread in the immune system and can be induced by several mechanisms; for example, the granule proteins released by cytotoxic T cells kill their target cells by inducing apoptosis. Another well-defined apoptotic pathway is that triggered by the interaction of the receptor molecule Fas with its ligand, called Fas ligand (FasL) (Fig. 19.2), which induces apoptosis in the Fas-bearing cell. FasL is a member of the tumor necrosis factor (TNF) family of membrane-associated cytokines, and Fas is a member of the TNF receptor (TNFR) family. Both Fas and FasL are normally induced on lymphocytes and other cell types during the course of an adaptive immune response. Apoptosis induced by cytotoxic T cells bearing FasL is a minor mechanism of cytotoxicity, whereas apoptosis in lymphocytes themselves, induced through Fas, seems to be an important mechanism of lymphocyte homeostasis, as this case shows. Finally, apoptosis can also be induced through a mitochondria-dependent mechanism (the so-called 'intrinsic pathway' of apoptosis), in which cell damage, cytokine deprivation, and other mechanisms result in an increased release of cytochome *c* contained in mitochondria, and the activation of caspase 9.

Topics bearing on this case:
Lymphocyte survival
Fas–Fas ligand interactions
Apoptosis
Lymphocyte activation
Autoimmune disease
TUNEL staining

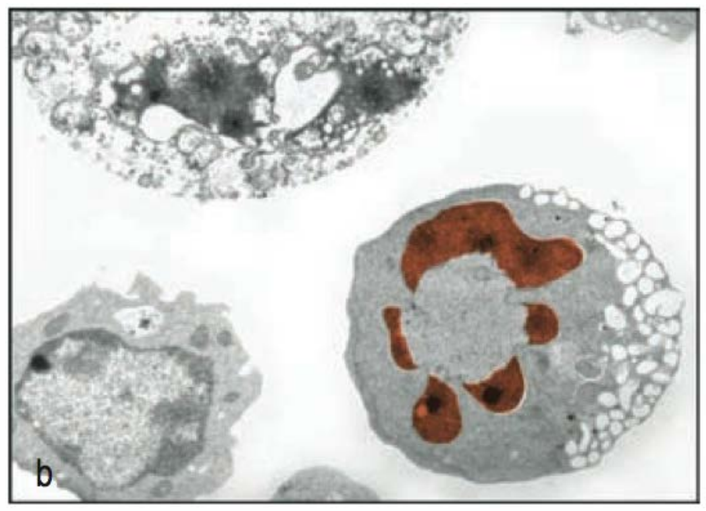
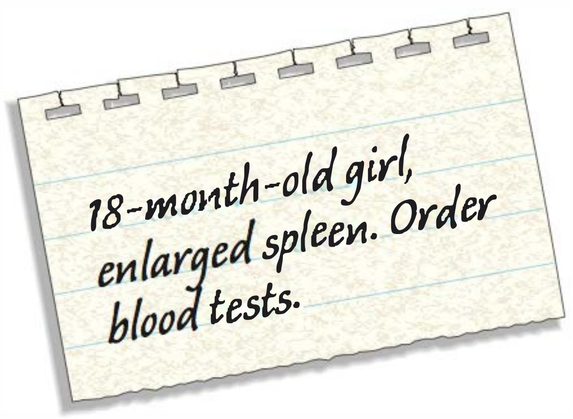


Fig. 19.1 Apoptosis. Apoptosis is a form of induced 'cell suicide' in which the cell undergoes chromatin compaction and DNA fragmentation, followed by cell shrinkage and internal degradation. Panel (a) shows a healthy cell with a normal nucleus. Early in apoptosis (panel b), the chromatin in the nucleus becomes condensed (red) and, although the cell sheds membrane vesicles, the integrity of the cell membrane is retained, in contrast to the necrotic cell in the upper part of the same field. In late stages of apoptosis (panel c), the cell nucleus (middle cell) is very condensed, no mitochondria are visible, and the cell has lost much of its cytoplasm and membrane through the shedding of vesicles. Photographs (× 3500) courtesy of R. Windsor and E. Hirst.



The case of Ellen O'Hara: uncontrolled lymphocyte proliferation in the absence of infection or malignancy.

Ellen O'Hara was born after a normal and uncomplicated pregnancy, was breast fed, and received her routine immunizations without any adverse reactions. At 18 months old, during a routine check-up by her pediatrician, she was found to have an enlarged spleen (splenomegaly) and extensive enlargement of her lymph nodes (lymphadenopathy) (Fig. 19.3). According to her parents, she had had no unusual infections and seemed to be growing and developing normally.

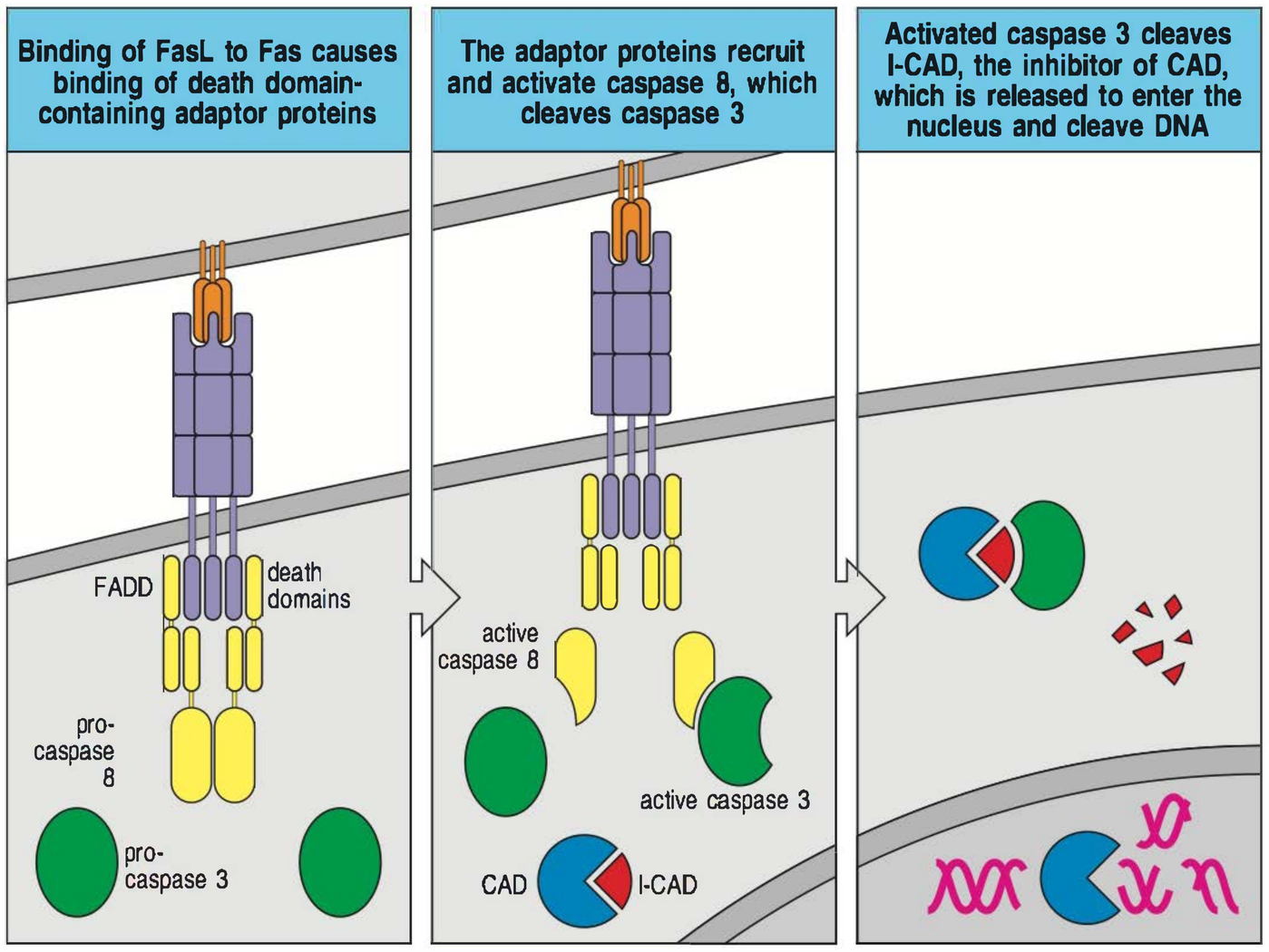


Fig. 19.2 Binding of FasL to Fas initiates the process of apoptosis in the Fas-bearing cell. Binding of trimeric FasL to trimeric Fas brings the death domains in the Fas cytoplasmic tails together. A number of adaptor proteins containing death domains bind to the death domains of Fas, in particular the protein FADD, which in turn interacts through a second death domain with the protease caspase 8. Clustered caspase 8 can transactivate, cleaving caspase 8 itself to release an active caspase domain that in turn can activate other caspases. The ensuing caspase cascade culminates in the activation of the caspase-activatable DNase (CAD), which is present in all cells in an inactive cytoplasmic form bound to an inhibitory protein called I-CAD. When I-CAD is broken down by caspases, CAD can enter the nucleus, where it cleaves DNA into the 200 bp fragments that are characteristic of apoptosis.

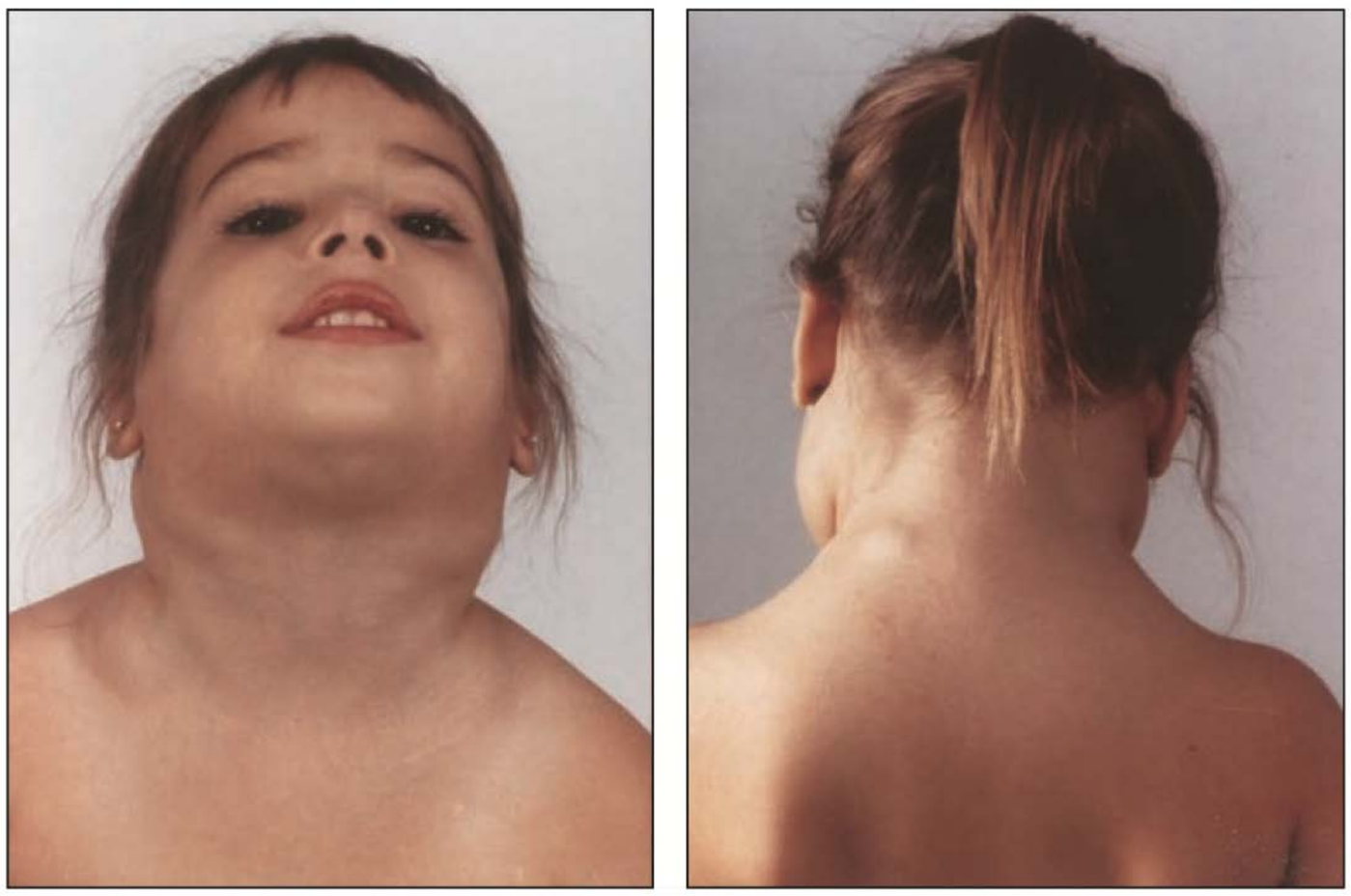
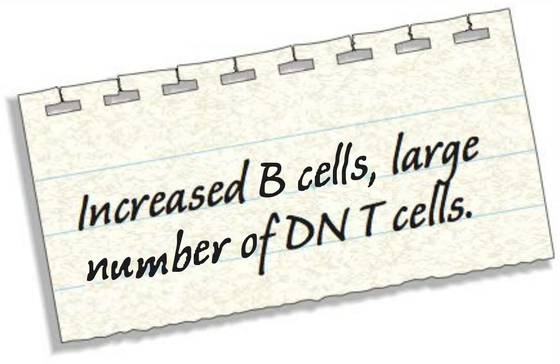
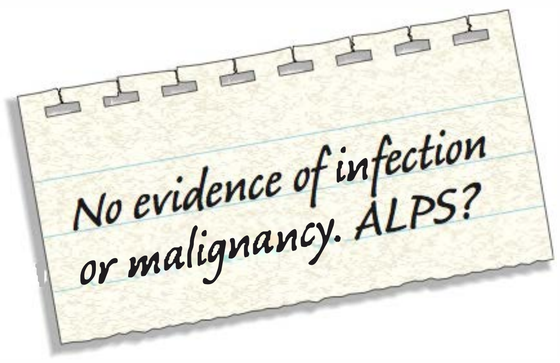


Fig. 19.3 Lymphadenopathy in ALPS. Young girl with ALPS with very enlarged lymph nodes in her neck. Photograph courtesy of Jennifer Puck.

Laboratory tests revealed that Ellen's white blood cell count was 12,500 μl^{-1}, of which 9175 were lymphocytes (normal 3000–7500). Her serum immunoglobulins were all elevated: IgG, 4000 mg dl^{-1} (normal 520–1500); IgM, 400 mg dl^{-1} (normal 40–200); and IgA, 1660 mg dl^{-1}. Flow cytometry analysis of her lymphocytes revealed that 29% were CD19-positive B cells (normal 5–15%; CD19 is a component of the B-cell co-receptor complex) and 65% were CD3-positive T cells (normal 61–84%; CD3 is a component of the T-cell receptor complex). Of the CD3-positive T cells, 14% carried the co-receptor protein CD4 and 18% the co-receptor CD8. She thus had many $CD3^+4^-8^-$ T cells. Of these, the vast majority expressed TCR$\alpha\beta$ (the $\alpha\beta$ form of the T-cell-receptors) and hence were TCR$\alpha\beta^+$ double-negative (DN) T cells (normally, TCR$\alpha\beta^+$ DN T cells are either absent or constitute less than 2% of circulating T cells). A biopsy of a lymph node from Ellen's neck showed extensive enlargement of the follicles (hyperplasia) and a marked increase in the numbers of immunoblasts and plasma cells in the paracortical area. No infectious agents were cultured from the lymph node, despite the fact that the observed changes resembled those caused by a viral infection. Although more than 50% of the T cells in the lymph node were double negatives, no chromosomal abnormality was found on karyotyping, and there was no evidence of oligoclonality of the T-cell receptor, thus ruling out a malignancy.

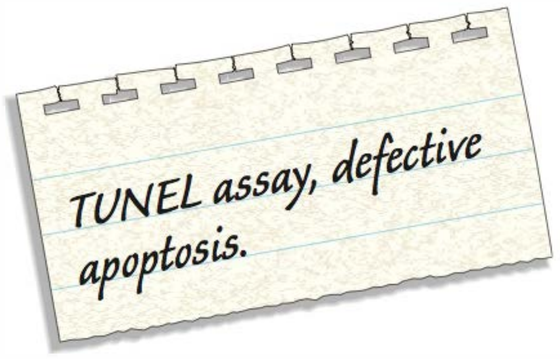


In the absence of evidence of infection or malignant disease, autoimmune lymphoproliferative disease was diagnosed and Ellen received the anti-inflammatory steroid prednisone and the immunosuppressant drug cyclosporin A. Her lymph nodes rapidly reduced in size after this therapy, but enlarged again when therapy was discontinued.



Ellen continued to grow and develop normally, and when she reached adolescence the size of her lymph nodes decreased spontaneously. At 18 years of age, repeat blood counts revealed that her platelet count was 75,000 μl^{-1} (normal 150,000–250,000). An autoantibody against platelets was found in her serum. A diagnosis of idiopathic thrombocytopenic purpura (low platelet numbers accompanied by red or purplish-red spotty skin discoloration due to local hemorrhages) was made. She was treated with the steroid dexamethasone, and the condition resolved. At age 32, Ellen's blood neutrophil count fell to <1000 μl^{-1} (normal 2500–5000). She was found to have developed an autoantibody against granulocytes.

Ellen's family history was informative in that her paternal grandfather had splenomegaly and generalized lymphadenopathy as a child, and his spleen was removed at age 25. At age 60, he developed a B-cell lymphoma. Ellen's father also had



splenomegaly and lymphadenopathy but no clinical symptoms. When blood lymphocytes from Ellen's father and paternal grandfather were examined by flow cytometry, a large number of double-negative T cells were found. In contrast, her brother, mother, and maternal grandparents had normal T cells. The TUNEL assay for apoptotic cells (Fig. 19.4) was performed on blood mononuclear cells from Ellen, her parents, and her paternal grandfather. The cells were first stimulated *in vitro* with phytohemagglutinin for 3 days, and growth of the resulting T-cell blasts was continued for 3 weeks by the addition of IL-2 to the cultures. The cultures were then divided; half were exposed to an antibody to Fas, which mimics the function of FasL. The percentage of cells undergoing apoptosis was then counted. Sixty percent of her mother's T cells underwent apoptosis, whereas only 2% of Ellen's cells, <1% of her father's cells, and 1.4% of her paternal grandfather's cells demonstrated programmed cell death (normal controls 35–70%). The *FAS* and *FASL* genes were examined in DNA samples from Ellen, her father, and her paternal grandfather. An identical single-base transversion, causing a premature termination codon, was found in one of the alleles of the *FAS* gene in these DNA samples. The *FAS* genes in Ellen's mother and brother were normal.

Autoimmune lymphoproliferative syndrome (ALPS).

ALPS is characterized by splenomegaly and lymphadenopathy from early childhood, and, frequently, autoimmunity. Affected individuals can develop autoimmune hemolytic anemia, neutropenia, thrombocytopenia, and hepatitis (inflammation of the liver) and are at increased risk of developing lymphoma. Most patients with ALPS are heterozygous for a dominant mutation in the *FAS* gene, and their activated T cells do not undergo Fas-mediated apoptosis *in vitro*, as is the case for Ellen and her father and grandfather. Patients with ALPS due to *FAS* mutations also have elevated serum levels of FasL, IL-10, and vitamin B_{12}; these can be used as reliable biomarkers, along with the increase in DN T lymphocytes. In some cases, ALPS is due to somatic mutations of *FAS* that occur in an early lymphoid progenitor. Because of the impairment of apoptosis, the proportion of lymphocytes carrying the somatic mutations may increase over time, and is particularly high among DN T cells.

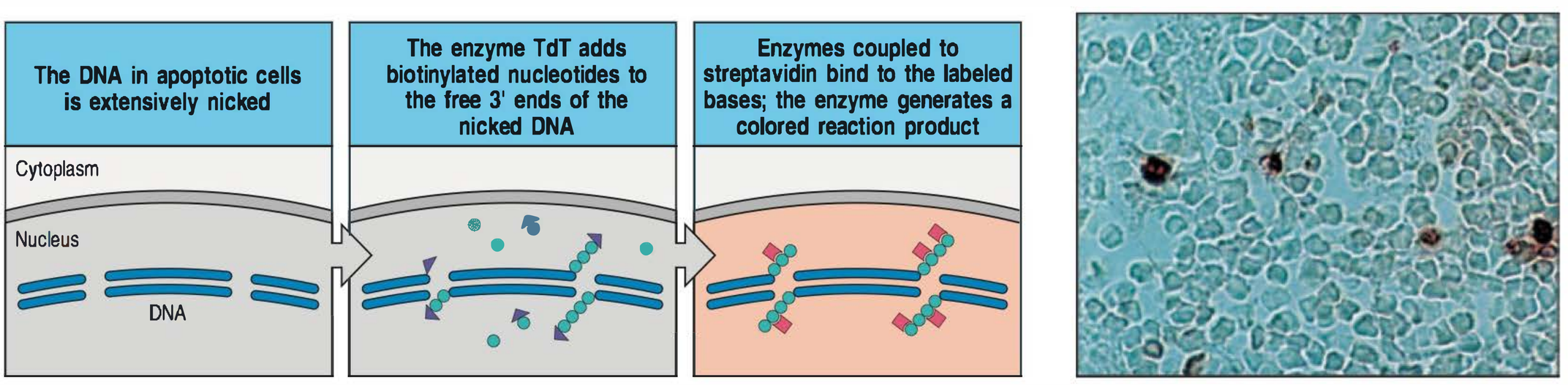


Fig. 19.4 The TUNEL assay. When cells undergo apoptosis, their DNA becomes fragmented and they can be revealed by labeling the fragmented DNA by using the enzyme terminal deoxynucleotidyltransferase (TdT). TdT adds nucleotides to the ends of DNA fragments; biotin-labeled nucleotides (usually dUTP) are most commonly added in this assay (second panel). The biotinylated DNA can be detected by using streptavidin, which binds to biotin, coupled to enzymes that convert a colorless substrate into a colored insoluble product (third panel). Cells stained in this way can be detected by light microscopy, as shown in the photograph of apoptotic cells (stained red) in the thymic cortex. Photograph courtesy of R. Budd and J. Russell.

Other patients with ALPS have been found to have mutations in the genes encoding FasL or caspase 10, an enzyme involved in triggering apoptosis via the Fas pathway. In one case, a gain-of-function mutation of the *NRAS* gene was identified that resulted in impaired induction of apoptosis in response to IL-2 deprivation. The *NRAS* mutation in this patient resulted in impaired induction of the pro-apoptotic molecule Bim, which controls mitochondrial stability upon cytokine deprivation.

Treatment of ALPS is mostly based on immune suppression and the surveillance of tumors. Splenectomy should be reserved for severe cases, because of the risk of infections by encapsulated bacteria (see Case 16).

The clinical and immunologic features of ALPS bear a striking resemblance to a lymphoproliferative disease observed in mice with *lpr* or *gld* mutations. The *lpr* phenotype results from the absence of Fas, whereas the *gld* phenotype is caused by a mutation in FasL. A progressive accumulation of DN T cells is observed in both these strains of mice (note that these circulating $CD3^+$ DN T cells should not be confused with the immature $CD3^-$ $CD4^-$ $CD8^-$ 'double-negative' thymocytes that are a normal stage of T-cell development in the thymus). The mice make antibodies against double-stranded DNA, similar to the situation in human systemic lupus erythematosus (see Case 37). Consistent with these findings in mice, patients with ALPS have defective T-cell apoptosis and abnormal accumulations of DN T cells. When B cells are activated, they also express Fas and become susceptible to Fas-mediated apoptosis. Thus, activated B cells in ALPS are not properly eliminated. The serum concentrations of immunoglobulins increase (hypergammaglobulinemia), the number of B cells is increased (B-cell lymphocytosis), and pathological autoantibody production ensues. Because T cells and B cells are not eliminated normally, patients with ALPS are predisposed to develop lymphomas. Autoimmunity may result because Fas-mediated killing is a mechanism for removing autoreactive B cells.

Questions.

1 *Patients with ALPS are heterozygous for the mutation in FAS or FASL; they have one normal allele and one mutant allele. How do you explain the dominant inheritance?*

2 *Ellen's great-aunt (her paternal grandfather's sister) was found to have the same FAS mutation as Ellen, yet she had no symptoms. How can this be explained?*

3 *It is advantageous for viruses to inhibit apoptosis so that the host cells in which they thrive do not get eliminated by apoptosis induced by recognition by cytotoxic T cells. How might a virus accomplish this?*

4 *When Fas is activated by FasL it associates with and activates caspase 8 (see Fig. 19.2). When the gene encoding caspase 8 is knocked out in mice, this proves to be lethal at the fetal stage. Would it be worthwhile to search for caspase 8 mutations in patients with ALPS when there is no mutation in FAS or FASL?*

Overview of the Fas-dependent apoptotic pathway (Extrinsic Pathway)

- Upon binding of a lymphocyte to the antigen that fits its receptor, it gets activated and starts to proliferate rapidly to vastly increase the number of lymphocytes specific for this antigen. This rapid increase in number is called clonal expansion and lasts for about 8 days.
- These newly formed cells are called active lymphocytes. They undergo their final differentiation into effector cells which can remove the antigen.
- After these effector cells eliminate the antigen, they are no longer activated by the antigen and thus undergo apoptosis. The following mechanism isn't the only mechanism for apoptosis but is especially important in lymphocytes death.

1) The **trimeric** protein **FasL** binds to its receptor **Fas** which is also **trimeric**.

2) This brings the **death domains** in the Fas cytoplasmic tails together (purple rods in the figure).

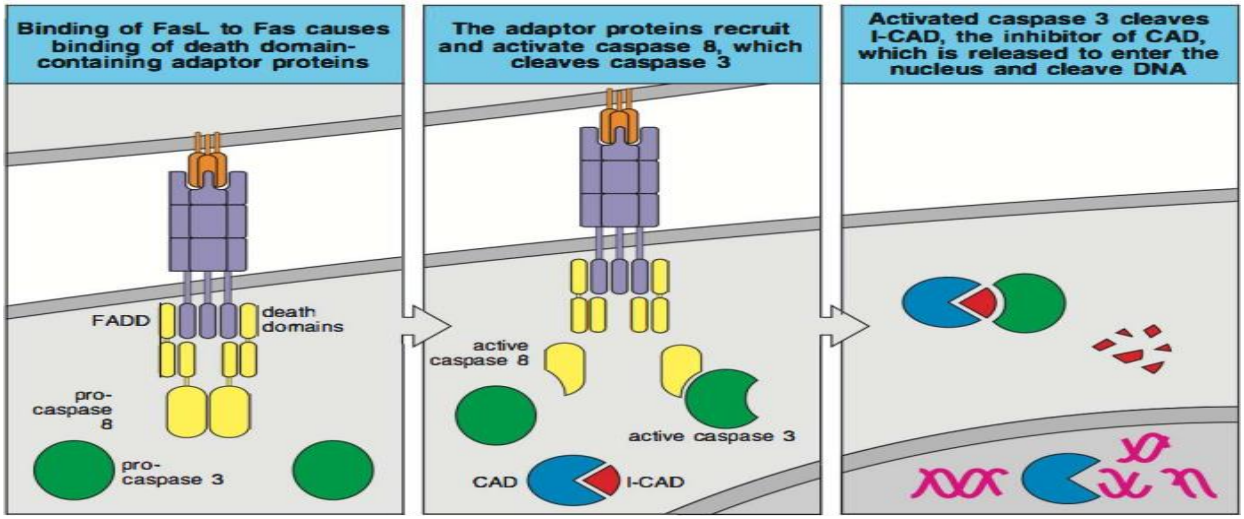
3) A number of adaptor proteins containing death domains bind to the death domains of Fas, in particular the protein **FADD** which has 2 death domains.

4) FADD interacts through the **second** death domain with the death domain of **pro-caspase 8** to produce the active protease, **caspase 8**.

5) Clustered caspase 8 can **transactivate**, cleaving caspase 8 itself to release an active caspase domain that in turn can activate other caspases such as **caspase 3**.

6) This cascade of caspases activating other cascpases continues and eventually, caspase 3 cleaves **I-CAD**.

7) This sets **CAD** free to enter the nucleus, where it cleaves the DNA into the **200 bp** fragments that are characteristic of apoptosis. ***CAD** (caspase-activatable DNase), is an enzyme present in all cells in an inactive cytoplasmic form bound to an inhibitory protein called **I-CAD**.



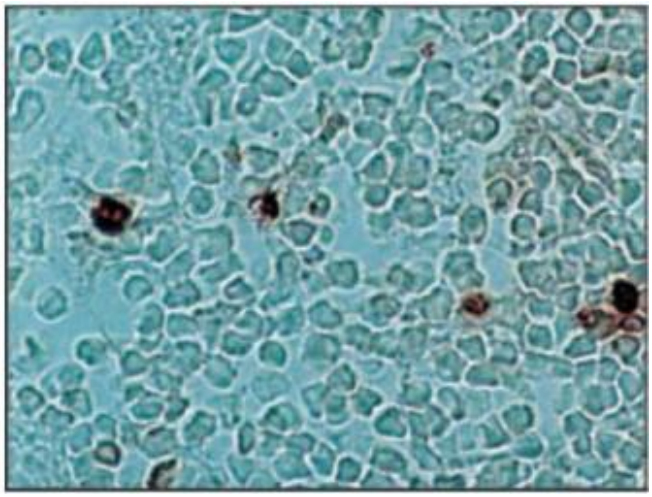
- **Notes regarding these molecules:**
  - Apoptosis is not accompanied with inflammation, in contrast to necrosis.
  - Fas is a membrane receptor of the TNF receptor family.
  - FasL is Fas ligand.
  - Both are induced on lymphocytes and other cell types during the course of an adaptive immune response.
  - FasL on cytotoxic T cells can bind Fas on infected cells to trigger apoptosis. However this mechanism for cytotoxicity isn't effective in getting rid of infected or cancerous cells.
  - Domain: a conserved part of a given protein sequence and tertiary structure that can evolve, function, and exist independently of the rest of the protein chain.

Autoimmune Lymphoproliferative Syndrome (ALPS)

- Apoptosis is essential in getting rid of activated immune cells, especially lymphocytes.
- Most cases of ALPS are heterozygous for a dominant mutation in the Fas gene (patients have the disease despite having one normal copy of the gene).
- Less numbers of ALPS patients have mutations in FasL or caspase 10 genes.
- This results in impaired apoptosis of lymphocytes and thus, unrestricted lymphoproliferation.
- This results in lymphocytosis (increased numbers of both T-cells and B-cells) which can progress into autoimmune diseases or even more seriously, into lymphoma.

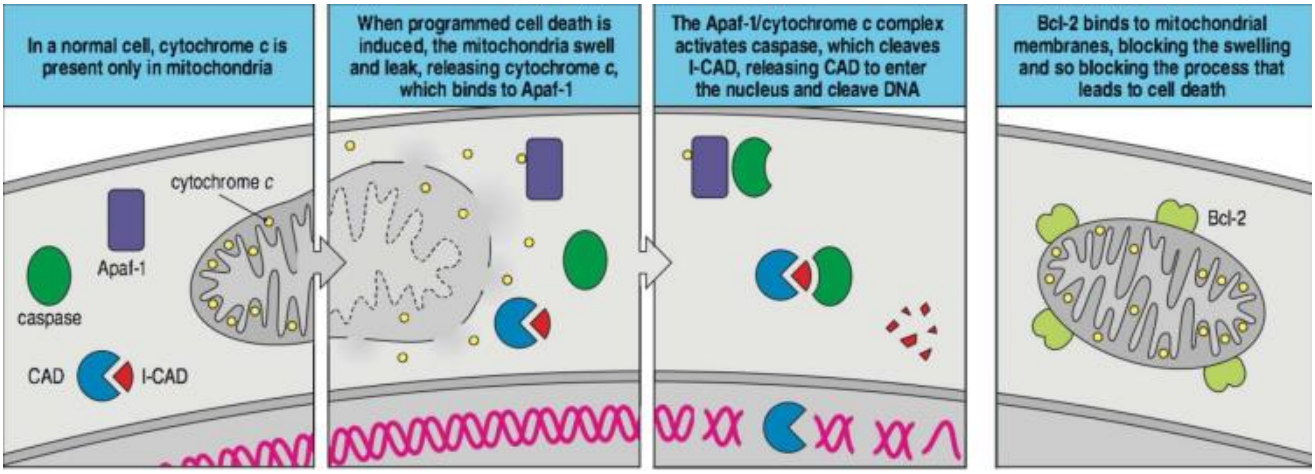
TUNEL Assay

- A technique frequently used in research labs to measure apoptosis.
- Normally, when the DNA in a cell gets nicked (fragmented), an enzyme called terminal deoxynucleotidyltransferase (TdT) adds nucleotides to the ends of DNA fragments to fill the gaps between the fragments. This is one of the cellular DNA repair mechanisms.



- In TUNEL, biotin-labeled nucleotides are added as substrates for TdT. When TdT joins these biotinylated nucleotides to the end of DNA fragments, biotin binds to streptavidin. This causes these colorless substrates to become colored under light microscope.
- If cells are undergoing apoptosis, high number of these fragments accumulating over each other will give rise to the "ladder" appearance.

Mitochondria-dependent Apoptotic Pathway (Intrinsic)



- This figure illustrates especially the importance of the anti-apoptotic protein Bcl-2 in inhibiting this pathway; it compresses the mitochondria blocking its swelling and release of cytochrome c.
- Caspase 8 in the extrinsic pathway is replaced by caspase 9 here.

Case of Ellen O'Hara

- She was 18 months old when symptoms started to appear.
  - This is consistent with the cause of the disease. Like any other diseases caused by genetic defects, symptoms start to appear early in life.
  - In these diseases, taking history of the patient becomes even more informative, and thus important in diagnosis.
- She presented with **splenomegaly, lymphadenopathy**.
  - The first possible diagnoses to think of when given such symptoms are infection and malignancy. How to exclude these two?
    - **Infections (Inflammation)**
- Lymph node biopsy to check for the presence of foreign microorganisms.

- C-reactive protein (**CRP**) level test. High CRP level indicates inflammation.
- Erythrocytes sedimentation rate (**ESR**) test. It will be high because of the increased level of fibrinogen which is involved in the repair process.

- **Malignancy (Mutations)**
  - Karyotyping, which reveals abnormalities in the number of chromosomes. Translocations and deletion may be difficult to detect so we rely on another technique:
  - Fluorescence in situ hybridization (**FISH**). A fluorescent probe (a fragment of the DNA that complement a specific site on the studied DNA) is added and its binding to DNA at: one site means that the studied DNA sample contains one copy of the gene or DNA segment; two sites means the DNA segment is duplicated; no sites at all means that the DNA segment is deleted. The site of binding may be on the wrong chromosome number, which means translocation of the DNA segment.
  - If the proliferation is malignant in such cases, then the produced T cells will arise from the same active T cell (i.e. all cells will possess the same TCR).

    - These tests were all negative in Ellen's case. Neither inflammation nor malignancy was proved.
    - After that, tests were performed to determine whether it was ALPS.
      - Lymphocytes are more sensitive to Fas dependent apoptosis. Therefore, blood test revealed increased lymphocytes count (T- and B-cell) and thus WBCs count was slightly elevated (13,000).
      - High B-cells count means increased secretion of antibodies. Immunoglobulin level test revealed elevated IgM, IgA, IgG.
      - Double negative (DN) T cells level is normally very low (less than 1% of all T cells). In **ALPS**, impaired apoptosis causes these cells to increase in number. Flow cytometry is a key technique in diagnosing ALPS. It revealed very high level of these cells (18%).
    - After ALPS was confirmed, Ellen received steroid and cyclosporine A (immunosuppressant). This treatment doesn't cure the disease; it only improves the symptoms and reduces lymph nodes size in almost all patients.

- Later, Ellen developed idiopathic thrombocytopenia purpura (**ITP**) as a result of formation of antibodies against her own platelets. This made her very prone to bleeding.
- She also developed antibodies against her own granulocytes which caused neutropenia. This caused her to suffer from recurrent infections.
- ALPS patients must be informed they are more susceptible to infections and unfortunately, lymphoma.

Questions:

1. How do you explain the dominant inheritance of ALPS?
2. Ellen's aunt had the same mutations as Ellen.
3. How can a virus benefit from inhibiting apoptosis in host cells?
4. How do viruses block apoptosis in host cells?
5. Caspase 8 gene knockout in mice is lethal, is it worth searching for mutations in Caspase 8 in ALPS patients with no mutaions in Fas or FasL?

Answers:

1. The function of the trimer protein Fas is very sensitive to any change in its structure. Therefore, any mutation in Fas gene results in complete (100%) loss of function.
2. In most genetically induced diseases, the genetic mutation itself is not sufficient to cause the disease but only increases the predisposition to the disease. First, the disease waits for the right environmental factors to trigger its appearance (just like autoimmune diseases wait for a trigger which is usually a serious infection). Second, the genetic mutation may require other unknown genetic factors that are necessary for the disease to appear. Ellen's aunt environmental factors and/or other genetic factors were different from those for Ellen.
3. Block ability of cytotoxic T cells to kill virally infected cells and thus maintain its replication.
4. **Vaccinia** *(belongs to poxvirus family)* and **HSV** produce molecules that block caspases and DNA fragmentation; EBV produces a Bcl-2-like protein which acts to prevent apoptosis, as discussed earlier.
5. Being important in mice doesn't mean loss of the function of this enzyme will also cause the human fetus to die. Interspecies differences are very enormous. 99% of the genome is homologous between humans and chimpanzee, for

example. Yet, these 2 species are very different in morphology and gene expression! Furthermore, caspase 8 has other Fas-independent functions. So, the answer is yes, we look for mutations in caspase 8.

Other useful points mentioned by the doctor but are not related to the case.

- The function of DN T cells is not fully understood yet.
- ESR increases in myeloma because increased Ig secretion causes RBCs agglutination.
- In myeloma, malignant B cells arise from one B cell (monoclonal). This means that secreted antibodies are all identical. Therefore, in electrophoresis, these antibodies accumulate in one site and form the M band which is considered diagnostic of the disease.
- Enzyme linked immunosorbent assay (ELISA) is a technique in which we can check for the presence of an antigen or an antibody in a sample. To check for the presence of an antibody, for example, the antigen is added to the sample and biotinylated fluorescent secondary antibodies are added and bind to the Fc portion of the primary antibodies which recognized the antigen if these primary antibodies are present. This binding gives a color that can then be analyzed.

More details about the case (not important)

Laboratory tests revealed that Ellen's white blood cell count was 12,500, of which 9175 were lymphocytes (normal 300-7500). Her serum immunoglobulins were all elevated: lgG, 4000 mg dl-1 (normal 52-1500); lgM, 400 mg dl-1 (normal 4-200); and lgA, 1660 mg dl-1• Flow cytometry analysis of her lymphocytes revealed that 29% were CD19-positive B cells (normal 5-15%; CD19 is a component of the 8-cell co-receptor complex) and 65% were CD3-positiveT cells (normal 61-400; CD3 is a component of the T-cell receptor complex). Of the CD3-positive T cells, 14% carried the co-receptor protein CD4 and 1 8% the co-receptor CDS. She thus had many CD3+4-s- T cells. Of these, the vast majority expressed TCR αβ (the αβ form of the T-cell-receptors) and

hence were TCR$\alpha\beta^+$ double-negative (ON) T cells (normally, TCR $\alpha\beta^+$ ON T cells are either absent or constitute less than 2% of circulating T cells). A biopsy of a lymph node from Ellen's neck showed extensive enlargement of the follicles (hyperplasia)

and a marked increase in the numbers of immunoblasts and plasma cells in the paracortical area.

Le Fin.

"You can only lose what you cling to, move on and be free..."

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Revised by: Omar Saffar