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Increased Expression of Fas Ligand in Human Tuberculosis and Leprosy Lesions: a Potential Novel Mechanism of Immune Evasion in Mycobacterial Infection

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To study the location and mechanism of apoptosis within the human tuberculosis (TB) and leprosy lesions, parallel sections were analyzed for mycobacterial antigens (M.Ag), Fas ligand (FasL), Fas, CD68 and Mac387 by immunohistochemistry, and apoptotic cells by the terminal deoxynucleotidyl-transferase-mediated dUTP-digoxigenin nick end labelling method. Cutaneous leishmaniasis and foreign body granulomas were analyzed for comparison. The heavily infected macrophages in multibacillary TB and leprosy granulomas very strongly expressed FasL, indicating that a mycobacterial infection can induce an increased expression of FasL in a population of infected macrophages, which may protect them from the attack of Fas-expressing lymphocytes. However, macrophages with high levels of leishmania amastigotes did not selectively express FasL, suggesting that this phenomenon is specific for the mycobacteria. Interestingly, in the well-formed TB granulomas, 84% of the multinucleated giant cells strongly expressed FasL. The expression of Fas was weak (34%) or absent. A higher number (33%) of epithelioid cells expressed FasL than Fas (23%). Lymphocytes were scanty among the epithelioid cells. The frequency of apoptotic cells was higher in the epithelioid cells (0.25%) than the mononuclear cells in the mantle zone (0.14%). Thus, the epithelioid cells and the multinucleated giant cells by virtue of the increased expression of FasL may make these granulomas an immune privileged site for mycobacteria.

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INTRODUCTION

Mycobacterium tuberculosis (*M. tuberculosis*) is an intracellular pathogen that preferentially resides within macrophages. The macrophage response to *M. tuberculosis* ultimately causes induction of cell-mediated immunity resulting in a gradual development of granuloma after infection [1]. T cells have protective roles in TB by virtue of secretion of cytokines, which will activate the macrophages and enhance their microbicidal capacity [1]. In spite of all the immune mechanisms mobilized in the host, mechanisms to survive the hostile environment in some macrophages have evolved in *M. tuberculosis* [2].

There is evidence from *in vitro* studies that *M. tuberculosis* on its interaction with human monocyte-derived macrophages [3,4], and human alveolar macrophages [5] can trigger apoptosis in its host cells. The apoptotic response to mycobacteria may be restricted to mature macrophages. The monocytes are shown to undergo spontaneous apoptosis in the absence of growth factors, but can be rescued by activating agents including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and *Escherichia coli* (*E. coli*) lipopolysaccharides (LPS) [6], as well as by infection with *Leishmania donovani* [7]. Virulent *M. tuberculosis* in low numbers is also shown to rescue human monocytes from apoptosis [8]. In contrast, differentiated macrophages lose their sensitivity to growth factor withdrawal and are susceptible to activation-induced apoptosis [9]. Thus, the regulation of

programmed cell death in terminally differentiated macrophages is distinct from that of their precursors *in vitro*. The situation might be very different *in vivo*. It can be hypothesized that the apoptosis of the host cells is an important defence mechanism and inhibition of apoptosis could favour *M. tuberculosis* by providing an intracellular sanctuary. Studies which showed that apoptosis of the infected macrophages but not lysis have a direct antimicrobial effect, support to this hypothesis [10,11]. Inverse correlation has been found between the virulence of *M. tuberculosis* and the apoptosis of the host cell. Virulent strains of *M. tuberculosis* are shown to be less potent inducers of apoptosis as compared to avirulent strains [5,12].

In order to propagate the infection, *M. tuberculosis* needs to keep its intracellular sanctuary. Cytotoxic T lymphocytes (CTL) are, however, supposed to kill the infected macrophages that can not rid themselves of the pathogen. CTL are shown to play an important role in the control of TB [11,13], and mycobacterial antigen specific CTL are shown to kill the infected macrophages *in vitro* [11]. However, *in vivo* in TB lesions the host CTL response seems unable to remove heavily infected macrophages.

Differentiated macrophages are shown to be a constitutive source of FasL in the human immune system [14], in addition to the activated lymphocytes. The macrophages in the murine lungs are also shown to express FasL constitutively [15]. The functional relevance of this apoptosis-inducing ligand in macrophages is unclear. In slowly progressive primary murine TB, we have recently shown that 5–7% of the vacuolated macrophages in the pulmonary lesions, which are heavily infected, selectively show an increased expression of FasL, and a reduced expression of Fas. The macrophage aggregates containing these vacuolated macrophages show very few lymphocytes. The rate of apoptosis is higher in the macrophage aggregates as compared to the lymphocyte aggregates. Based on these findings we postulated that *M. tuberculosis* infection could induce an increased expression of FasL in a population of infected macrophages. As a consequence, the infected macrophages can be protected from the attack of CTL. In fact, they could kill nearby lymphocytes and thus avoid the activation of bactericidal mechanisms by sensitized lymphocytes. This constitutes a novel evasion mechanism for *M. tuberculosis* in mice [15].

Following these murine studies, the aim of the present studies was to examine the *in situ* pattern of apoptosis in biopsies from human TB with a particular focus on the Fas and FasL expression. In addition, specimens from multibacillary and paucibacillary leprosy, cutaneous leishmaniasis and foreign-body granulomas were compared.

MATERIALS AND METHODS

Biopsy material. Biopsy specimens for the four granulomatous conditions, namely TB, leprosy, cutaneous leishmaniasis, and foreign-body granulomas were made available from Armauer Hansens Research Institute, Addis Ababa, Ethiopia; Haukeland University Hospital, Bergen, Norway; and Instituto Nacional de la Nutricion, Salvador Zubiran, Mexico City, Mexico. Twenty-five biopsies diagnosed as TB were selected. Twenty-three biopsies were from the lymph nodes, one from the skin, and one from the liver. The diagnosis was made on the basis of histopathological findings typical for TB granulomas and the presence of acid fast bacilli in some of the samples. Both acid fast staining and immunohistochemistry were used to study the presence of bacilli. The immunohistochemical method was preferred because it was easy to find the positive signals at a lower magnification. Eleven leprosy biopsy specimens from skin or nerve, four cutaneous leishmaniasis biopsy specimens, and six biopsies from skin diagnosed as foreign-body granulomas, were selected. All the biopsies were fixed in 10% buffered formalin and routinely embedded in paraffin.

Tissue preparation. Parallel 5 μm thick sections, seven in each set, were prepared from each specimen. The first section was placed on an untreated glass slide for haematoxylin and eosin (H&E) staining. The subsequent sections were placed on glass slides pretreated with 0.01% aqueous solution of poly L-lysine (300 000 mol.wt., Sigma Chemical Co., St. Louis, MO, USA) for immunohistochemical staining and terminal deoxynucleotidyl-transferase-mediated dUTP-digoxigenin nick end labelling (TUNEL). Sections were heated overnight at 37 °C and stained in the following order: firstly, H&E staining; secondly, a-bacille Calmette–Guérin (BCG). In order to detect multiple antigens of *M. tuberculosis* and *M. leprae* in the tissues, polyclonal antibody reacting with multiple antigens of sonicate and culture fluid of *M. bovis* BCG was used. *M. bovis* BCG and *M. tuberculosis* are extensively similar in their antigenic content [16]. Anti-BCG is expected to react with multiple antigens of *M. tuberculosis* and *M. leprae* [17]. The antigens detected by a BCG were designated as mycobacterial antigens (M.Ag); thirdly, FasL detection; fourthly, Fas detection; fifthly, TUNEL for apoptotic cells; sixthly CD68; seventhly, Mac387. All the antibodies used are described in Table 1.

Immunohistochemistry. The procedure was described previously [15]. The sections were briefly treated with 0.1% trypsin at 37 °C for 20 min for antigen retrieval after deparaffinization and rehydration. Endogenous peroxidase activity was blocked by treatment with H_2O_2 and endogenous biotin activity by avidin and biotin blocking solution (Vector Laboratories, Burlingame, CA, USA). After incubation with normal serum of the animal species in which the secondary antibody was made, the sections were incubated overnight at 4 °C with different primary antibodies. All the primary antibodies used were diluted in 1% v/v bovine serum albumin (BSA) (Calbiochem, La Jolla, CA, USA)/ Tris phosphate-buffered saline (TBS) solution. Incubation with biotinylated secondary antibodies was done for 60 min followed by a 60 min incubation with avidin-biotin-peroxidase complex (ABC) (Dako A/S, Glostrup, Denmark) at room temperature. Location of antigens was visualized by a H_2O_2 and 3-amino-9-ethylcarbazol containing buffer for 15 min. The slides were washed in TBS between each step and slightly counterstained with Mayer's haematoxylin. We used 1% BSA/TBS instead of primary antibody, and an irrelevant antibody from the same species, with the same Ig class as the primary antibody as the negative control.

TUNEL method. The TUNEL method was used to visualize apoptotic cells *in situ* [18]. Sections were deparaffinized, rehydrated, and pretreated with 20 μg/ml of proteinase K (Medinor AS, Oslo, Norway) to strip the nuclei of tissue section from proteins. In order to neutralize the endogenous peroxidase the sections were preincubated with 0.1% H_2O_2 in TBS for 30 min at room temperature and then washed with TBS. In order to label the apoptotic cells the sections were washed twice for 5 min with terminal transferase buffer (TdT-buffer) at 37 °C (0.5 M cacodylate, pH 6.8, 1 mM $CoCl_2$, 0.5 mM dithiothreitol

Table 1. Antibodies used in the immunohistochemical staining

Antibodies	Specificity	Source
Rabbit polyclonal	Multiple antigens of *M. bovis* BCG Copenhagen strain	Dako A/S, Denmark
Rabbit polyclonal	Fas Ligand	Santa Cruz Biotechnology, USA
Rabbit polyclonal	Fas	Santa Cruz Biotechnology, USA
Mouse monoclonal IgG1, KP1	CD68 expressed on Monocytes/macrophages	Dako A/S, Denmark
Mouse monoclonal IgG1, Mac387	Calprotectin/L1 antigen expressed on monocytes/macrophages	Dako A/S, Denmark
Rabbit Igs	Reacts with mouse Igs of all classes	Dako A/S, Denmark
Swine Igs	Reacts with rabbit Igs of all classes	Dako A/S, Denmark

(DTT), 0.05% BSA and 0.15 M NaCl), and then covered with TdT-buffer containing four units TdT (Boehringer Mannheim, Mannheim, Germany) and 2 μ M digoxigenin-conjugated dUTP (Boehringer Mannheim) and incubated at 37 °C for 1 h. After washing once with TBS and once with TBS supplemented with 2% fetal calf serum (FCS), the sections were incubated with sheep antidigoxigenin (Boehringer Mannheim) diluted in 2% FCS/TBS for 1 h at room temperature, washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit antisheep IgG (5 μg/ml, Dako A/S) diluted in 2% FCS/TBS for 1 h at room temperature. After washing, the bound HRP was detected by incubation with H_2O_2 and 3-amino-9-ethylcarbazol containing buffer for 15 min, washed and slightly counterstained with Mayer's haematoxylin. After washing in running tap water for 5 min the sections were mounted with Immunomount (Pittsburgh, PA 15275, USA).

Evaluation of immunostaining and TUNEL. Cell counts were performed using a light microscope and a × 40 ocular fitted with a 10 × 10 mm graticule. The entire multinucleated giant cells (MGC) in all the granulomas in a section were counted. Stained MGC with FasL or Fas were counted and described as the percentage of all MGC. For all other cells 3–5 fields and for apoptotic cells 6–11 fields were randomly selected within the lesions and counted. The stained cells, apoptotic cells and the total number of nucleated cells were counted for each field. The percentage of the stained cells and the apoptotic cells designated as apoptotic index (AI) was calculated.

Statistical analysis. Friedman's Test, Wilcoxon's signed-rank test and Mann–Whitney's *U*-test were used.

RESULTS

Morphology and mycobacterial antigens in TB and leprosy granulomas

TB biopsies were divided into two groups depending on the histopathology of the lesions and the capacity of the a-BCG antiserum to detect M.Ag. Twenty two biopsies consisted mainly of well-formed layers of epithelioid cells with MGC, which surrounded the central areas of necrosis. There was an outer zone of lymphocyte-like cells, referred to as mononuclear cells in the text, and fibrosis. These biopsies contained no detectable M.Ag.

The other group consisted of three TB cases. The granulomas were not organized and consisted essentially of vacuolated macrophages mixed with mononuclear cells and few polymorphonuclear cells. No epithelioid cells or MGC were seen. M.Ag were detectable in the vacuolated macrophages in these biopsies. The heavily infected macrophages were lying in aggregates. These biopsies were referred as multibacillary TB. These two types of histological appearance of TB lesions are similar to those observed in the tuberculoid and the lepromatous part of the histopathological spectrum of leprosy, as described previously [19].

The leprosy biopsies were divided into two groups according to the number of M.Ag positive cells and the amount of antigens in these cells (Table 2). One group constituted seven cases of multibacillary leprosy. These lesions had a high amount and number of M.Ag positive vacuolated macrophages. In the other group, which constituted four paucibacillary biopsies, the lesions contained a very small amount and number of M.Ag-containing cells.

Multibacillary TB and leprosy granulomas: colocalization of mycobacterial antigens and FasL in the same group of cells

Table 2 shows the percentage of cells stained with M.Ag, FasL and Fas in the multibacillary TB granulomas. M.Ag were found in 11–30% of the vacuolated macrophages. The parallel section showed that these M.Ag containing macrophages expressed high amounts of FasL (Fig. 1A,B). The cells without M.Ag either did not express FasL or did expressed it, however at a very low level. The percentages of FasL and M.Ag-expressing cells from the same biopsy were rather similar (Table 2). The expression of

Table 2. Percentage of cells expressing M.Ag, FasL, Fas and the apoptotic index in the MB TB, leprosy and cutaneous leishmaniasis lesions

	Percentage of positive cells			
Diagnosis	M.Ag	FasL	Fas	AI
MB TB: skin	30	24	94	0.11
MB TB: liver	14	43	92	0.26
MB TB: lymph node	11	12	30	0.23
MB leprosy: skin	50	77	25	0
MB leprosy: skin	5	7	ND	0.19
MB leprosy: skin	5	9	19	0.15
MB leprosy: skin	9	16	29	0
MB leprosy: skin	3	7	97	0.025
MB leprosy: nerve	5	17	ND	0.75
MB leprosy: nerve	4	6	ND	0.24
PB leprosy: skin	0.8	8	97	0.025
PB leprosy: skin	0.4	2	25	0.34
PB leprosy: skin	0	ND	11	0
PB leprosy: skin	0	8	97	0
	Amastigotes	FasL	Fas	
DCL	+ + +	13	48	0.22
LCL	+	14	ND	0.01
LCL	+ – –	1	63	0.03
LCL	+	19	48	0.03

MB = multibacillary, PB = Paucibacillary, DCL = Diffuse cutaneous leishmaniasis, LCL = Localized cutaneous leishmaniasis, ND = Not counted due to weak and diffuse staining. Each row indicates results from one specimen. Cell counts were performed using a light microscope and a × 40 ocular fitted with a 10 × 10 mm graticule. For apoptotic cells 6–11 fields and for all other cells 3–5 fields were randomly selected within the lesions and counted. The stained cells, apoptotic cells and the total number of nucleated cells were counted for each field.

Fas was weak on the M.Ag-expressing macrophages. The amount of M.Ag and FasL (assessed by the intensity of staining) in the stained cells varied, with some cells fully loaded while other had lesser amount (Fig. 1A,B). Usually the cells with a higher intensity of M.Ag expression had higher intensity of FasL expression as well.

Similar observations were done in the multibacillary leprosy granulomas. Vacuolated macrophages with high amounts of M.Ag colocalized with vacuolated macrophages which expressed FasL strongly, both in skin (Fig. 1C,D) and nerve sections (Fig. 1F,G). In the nerve, the M.Ag and FasL staining was mainly in some cells in nerve bundles, a fact which makes it likely that these were Schwann cells. In the nerves, the expression of Fas was diffuse and weak and the positive cells could not be counted (Fig. 1H). In the skin lesions, the Fas and FasL (Fig. 1D,E) expression was equally strong. Table 2 shows the percentage of M.Ag, FasL, and Fas-positive cells in the multibacillary leprosy lesions.

Apoptotic cells were detected in both leprosy and TB granulomas. Table 2 shows the percentage of apoptotic cells in these lesions. The nerve lesions seemed to have a higher frequency of apoptotic cells than the skin lesions (Tables 2 and Fig. 1I). There was no clear association between apoptotic cells and FasL, Fas, or M.Ag-expressing cells.

The vacuolated macrophages in both TB and leprosy granulomas expressed the macrophage markers CD68, and Mac387.

Paucibacillary leprosy granulomas

Table 2 shows the percentage of M.Ag, FasL, Fas positive and apoptotic cells in the lesions of paucibacillary leprosy. M.Ag were either detected in $< 1\%$ of the macrophages, or not detected at all. FasL was expressed in 2–8% of the cells. The expression of FasL was not as strong as in the multibacillary granulomas. The macrophages expressed both CD68 and Mac387. Cells expressing CD68 were more frequent than Mac387-expressing cells.

In the skin, all the keratinocytes expressed Fas and FasL, except the basal layer, which did not express FasL. Sweat glands were stained with Fas but not FasL. In the hair follicles, mainly the outer root sheath was stained with Fas in all the biopsies. Staining of the hair follicles with FasL was present in 50% of the paucibacillary biopsies and in 75% of the multibacillary biopsies.

Cutaneous leishmaniasis

Cutaneous leishmaniasis lesions were analyzed for the expression of FasL in the macrophages containing amastigotes of *Leishmania aethiopica*. Table 2 shows the percentage of cells expressing FasL and Fas, and the apoptotic cells in the lesions of localized cutaneous and diffuse cutaneous leishmaniasis. The majority of cells expressing FasL were macrophages. The macrophages containing amastigotes did not selectively overexpress FasL.

Well-formed TB granulomas with no detectable mycobacterial antigens

Fas ligand. Figure 2 shows the percentage of cells expressing FasL in the group of well-formed TB granulomas. Eighty-four percent of the MGC and 33% of the epithelioid cells expressed FasL. Some MGC very strongly expressed FasL compared to other MGC, whereas the intensity of staining was uniform on epithelioid cells (Fig. 3A,C). Eighteen percent of the mononuclear cells in the mantle zone expressed FasL. The stain was mainly along the membranes on these cells, whereas in MGCs and epithelioid cells the stain was in the cytoplasm as well. A significantly higher number of epithelioid cells expressed FasL as compared to mononuclear cells ($P = 0.001$).

Fas. Figure 2 shows the percentage of cells expressing Fas. Fas was detected on only 34% of the MGC. A significantly higher number of MGC expressed FasL as compared to Fas

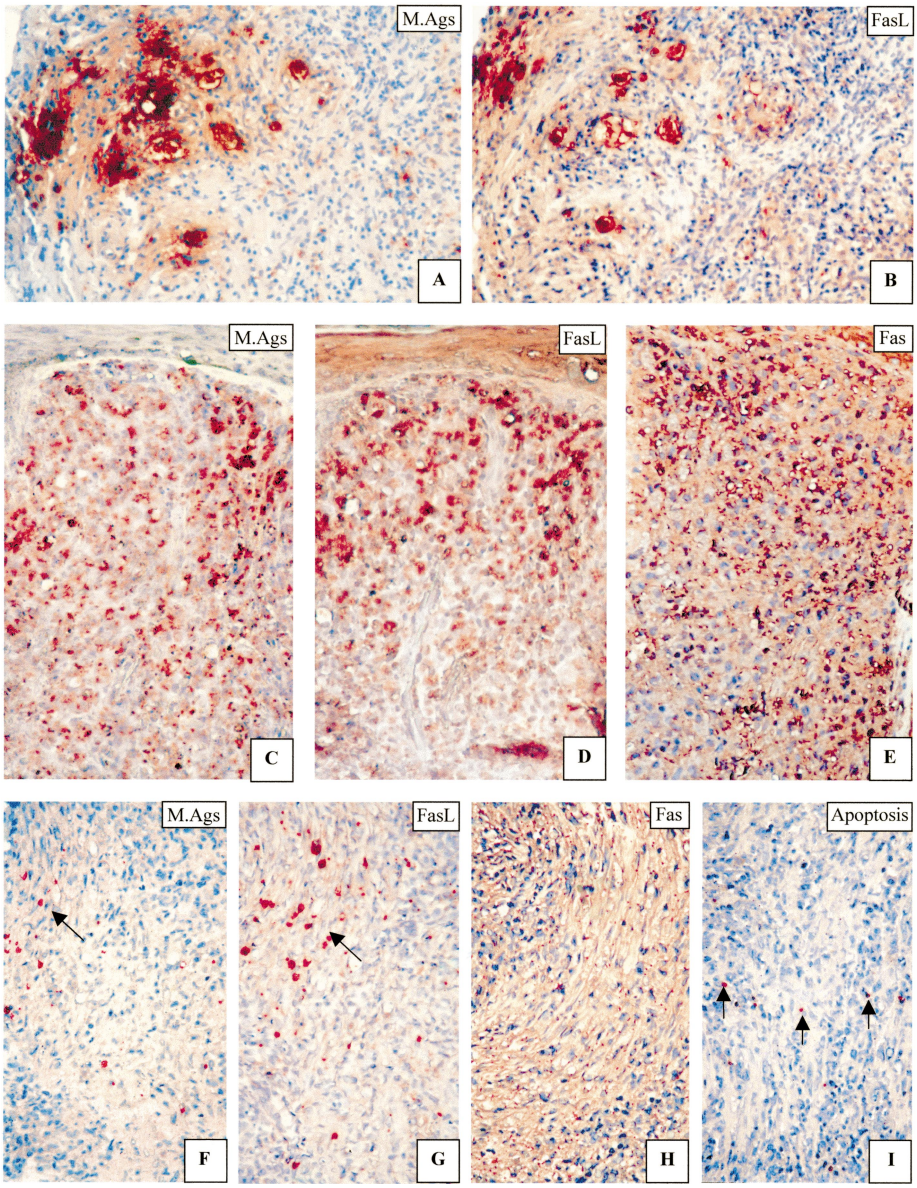

Fig. 1. The expression of mycobacterial antigens (M.Ag), Fas ligand (FasL), and Fas in the TB and leprosy lesions detected by immunoperoxidase staining with ABC, and apoptotic cells in leprosy lesion detected by TUNEL. (A–B) (× 200); TB granulomas. M.Ag were present in vacuolated macrophages. Some cells had a very high amount of antigens as compared to other cells. Parallel section showed that the same cells, which contained mycobacterial antigens, very strongly expressed FasL. (C–E) (× 200); Multibacillary leprosy of the skin. The majority of the vacuolated macrophages expresses M.Ag. These cells colocalized with cells expressing high amounts of FasL. Fas was also expressed by these vacuolated macrophages. (F–I) (× 200); Multibacillary leprosy of the nerve. The cells present in nerve bundles (arrows) containing M.Ag seemed to colocalize with the cells with strong expression of FasL. Fas expression was distributed on all the cells. Expression of Fas was weaker in the nerve as compared to the lesion in skin. Apoptotic cells in the same lesion were detected by TUNEL.

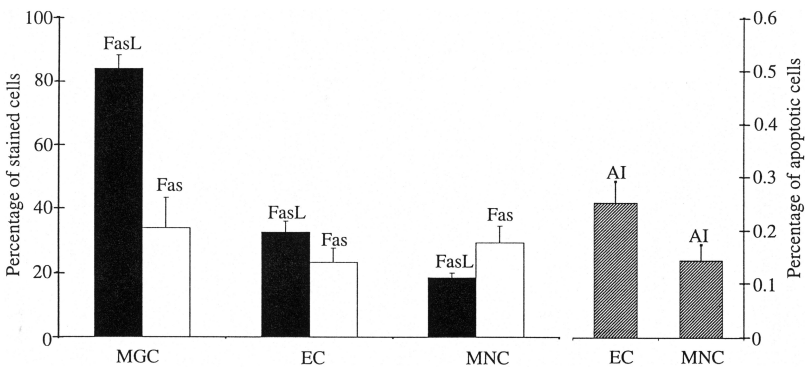

Fig. 2. Percentage of Fas ligand (FasL) and Fas positive, and apoptotic cells in the TB granulomas without detectable M.Ag, analyzed by immunohistochemical staining and TUNEL method ($n = 22$). Standard error of mean is marked. Number of FasL-expressing cells was higher than Fas-expressing cells amongst the mulinucleate giant cells (MGC) ($P = 0.001$) and epithelioid cells (EC) ($P = 0.02$). In the mononuclear cells (MNC), the number of FasL-expressing cells was lower than the Fas-expressing cells ($P = 0.02$). The apoptotic index (AI) was higher in the epithelioid cells as compared to the mononuclear cells ($P = 0.001$).

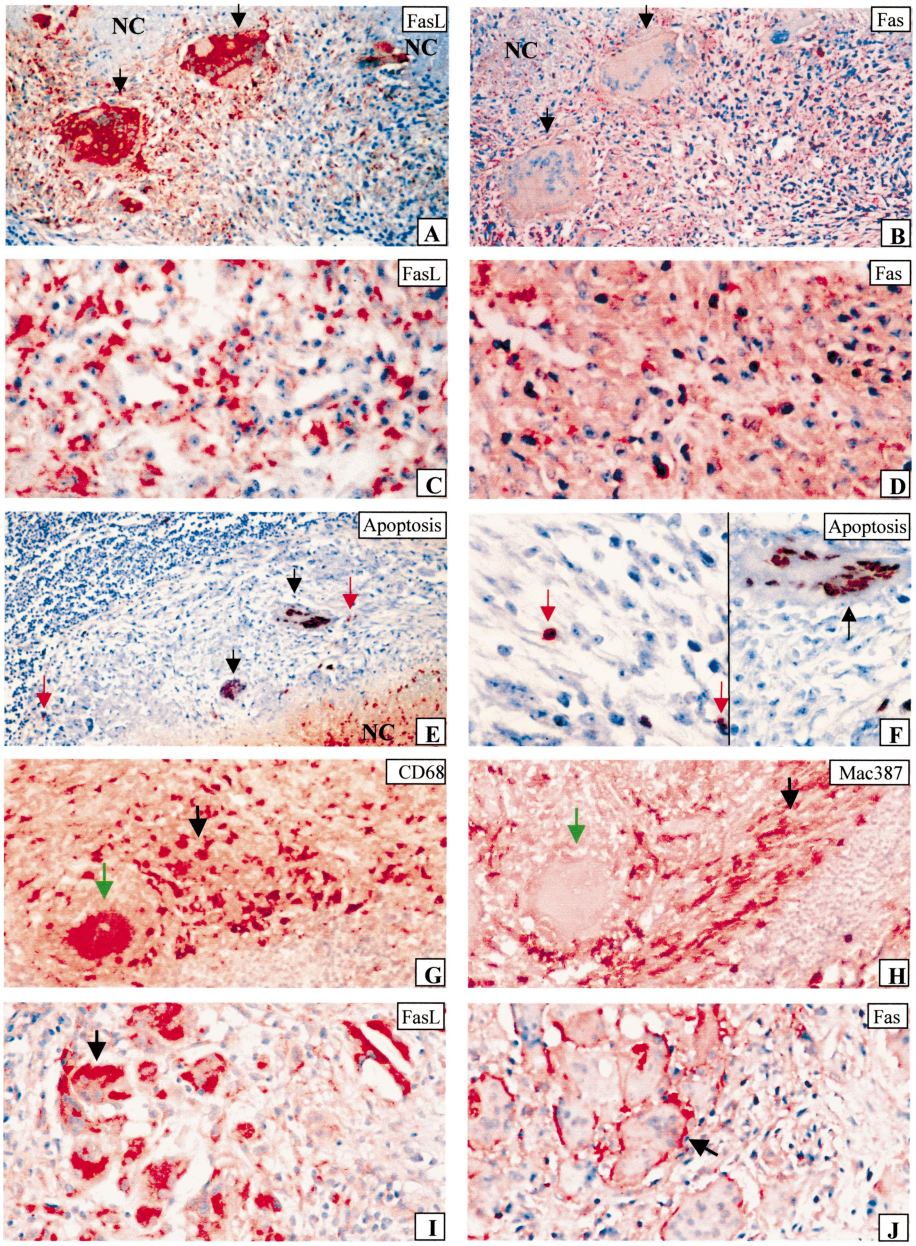

Fig. 3. Expression of Fas ligand (FasL), Fas, CD68, Mac387 and apoptotic cells in the well-formed TB (A–H) and foreign body (I–J) granulomas. (A–B) (× 120) the multinucleated giant cells (MGC) expressed FasL very strongly as compared to Fas. (C–D) (× 224) The epithelioid cells express both FasL and Fas. E × 112-F × 360. TUNEL staining showed the nuclei in MGC in apoptosis (black arrows). Apoptotic cells were seen in the epithelioid cells as well (red arrow). The necrotic centres (NC) were stained with the TUNEL staining as well. The nuclei around the necrotic cells were not included in the counting for apoptotic cells. (G–H) (× 160) Epithelioid cells (black arrow) expressed both CD68 and Mac387, but the MGC expressed CD68 but not Mac387 (green arrow). (I –J) (× 180); Foreign body granulomas. The MGC (arrows) expressed both FasL and Fas. The expression of FasL was cytoplasmic, while Fas was more restricted to the cell membranes. The epithelioid cells also expressed both FasL and Fas.

($P = 0.001$). The intensity of the expression of Fas was weaker as compared to FasL on MGC (Fig. 3A,B). In six cases the MGC which expressed FasL weakly expressed Fas, as detected by staining the consecutive sections.

About 23% of the epithelioid cells were stained for Fas (Fig. 3D). The number of epithelioid cells expressing FasL was significantly higher as compared to Fas ($P = 0.02$). Twenty-nine percent of the mononuclear cells in the mantle zone expressed Fas. Here the number of FasL-expressing cells was lower as compared to the Fas-expressing cells ($P = 0.03$).

Apoptosis. Figure 2 shows the percentage of apoptotic cells in the TB granulomas. Examples of the morphological appearance of the apoptotic cells are shown in Fig. 3(E,F). The frequency of apoptotic cells was significantly higher among the epithelioid cells than the mononuclear cells in the mantle zone ($P = 0.02$). The nuclei in the MGC were stained with TUNEL in one case only (Fig. 3E,F). Morphologically two modes of cell death, namely apoptosis and necrosis, were observed with the TUNEL stain. The necrotic centres were strongly stained (Fig. 3E). The areas immediately around the necrotic centres had a high number of stained cells. These areas were not included in the counting for AI as these cells were assumed to be necrotic rather than apoptotic [20].

CD68 and Mac387. Thirteen cases were studied for the expression of Mac387 and CD68. Epithelioid cells expressed both CD68 and Mac387, whereas MGCs expressed only CD68 (Fig. 3G,H). Among the mononuclear cells in the periphery of granulomas, some cells expressed these markers, suggesting that

that they are monocytes. Among the epithelioid cells more cells expressed CD68 than Mac387, whereas among the mononuclear cells more cells expressed Mac387 than CD68. In five out of 13 cases (38%) the epithelioid cells did not express Mac387.

Foreign body granulomas

FasL was detected on 79% of the MGC and 30% of the epithelioid cells as shown in Fig. 4. The epithelioid cells were scarce in these granulomas and the stain was localized in the cytoplasm. The stained cells had uniform intensity of staining (Fig. 3I).

Fas was detected on 74% of the MGC, and 22% of the epithelioid cells (Fig. 4). On the MGC, the pattern of staining was more along the membranes (Fig. 3J).

Frequency of apoptotic cells was higher in epithelioid cells (AI = 0.27) compared to the mononuclear cells (AI = 0.01) ($P = 0.07$) (Fig. 4).

CD68 was expressed on both epithelioid cells and MGC. Mac387 was expressed on epithelioid cells though with lesser frequency than CD68. MGC did not express Mac387.

Comparison of TB with the foreign-body granulomas

In the foreign-body granulomas a higher percentage of MGC expressed Fas (74%), as compared to TB granulomas (34%). The epithelioid cells expressed both FasL and Fas in both types of granulomas. The AI was higher in the TB granulomas compared to the foreign-body granulomas ($P = 0.005$). MGC in both granulomas expressed CD68 but not Mac387.

DISCUSSION

In human multibacillary leprosy and TB granulomas, the vacuolated macrophages containing an increased amount of M.Ag colocalized with macrophages expressing high amount of FasL as shown by staining of consecutive sections at 5 μm distance. This corresponded with the findings we have reported in murine TB lesions [15]. It was difficult to perform double staining on the same section because of the polyclonal antibodies used. We suggest that these heavily infected FasL-expressing macrophages provide the mycobacteria with an intracellular sanctuary from the T-cell surveillance by inducing apoptosis in T cells that express Fas. Other infectious agents such as the Epstein-Barr virus [21,22] and human immunodeficiency virus (HIV) [14,23], have also been shown to increase the expression of FasL on the cells they reside in, and can induce apoptosis in the Fas-expressing lymphocytes. Furthermore, constitutive FasL expression has also been detected on some tumour cells probably inducing apoptosis in Fas-expressing immune cells when they attempt to enter the tumour tissue [24]. Our results from the present study and a previous study on murine TB [15] show that this mechanism of immune evasion seems to be exploited by mycobacteria as well. Recently it has been shown that the *M. avium* infection in C57/BL6 mice renders CD8+ T cells prone to apoptosis when exposed *ex vivo* to macrophages infected with *M. avium* [25], which indirectly lends support to our hypothesis. The macrophages containing high numbers of leishmania amastigotes, however, did not selectively over-express FasL in our study, which implies that all infectious agents do not share this mechanism.

In our study FasL was expressed intracellularly in addition to its expression on the membranes. In fact it has been shown that after synthesis the FasL protein is maintained as intracellular stores in normal and tumoural monocytes, that are only released after cellular activation [26]. It has been shown that FasL is cleaved from the cell surface via a metalloproteinase, releasing an active, soluble form of the molecule [27,28]. Although the soluble FasL is said to be a less potent inducer of apoptosis as compared to the membrane associated FasL [29–31], it is shown to be functionally active against cells that are highly sensitive to Fas-mediated apoptosis [30,31]. Soluble FasL has in fact been detected in the sera of the patients with lymphoid [32] and solid tumours [33]. Whether soluble FasL is present in the sera of TB patients and has any systemic effects will be investigated in our laboratory.

Organization of the granuloma and the presence of epithelioid cells and MGC in the TB and leprosy lesions indicate the ability of the host to mount an effective immune response, capable of containing mycobacterial infection [34,35]. These are considered to be the consequence of an effective T-cell-mediated response [34,36]. In our study the group of TB biopsies where

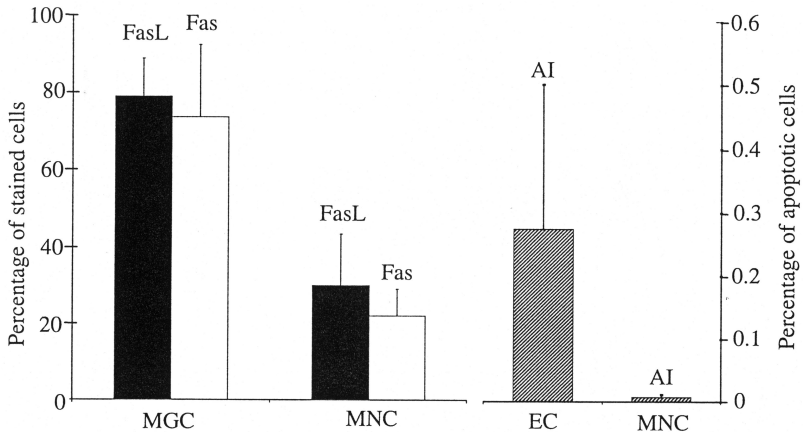

Fig. 4. Percentage of Fas ligand (FasL) and Fas positive, and apoptotic cells in the foreign body granulomas, analyzed by immunohistochemical staining and TUNEL method ($n = 6$). Bars indicate the standard error of mean. MGC = Multinucleated giant cells, EC = epithelioid cells, MNC = mononuclear cells, AI = Apoptotic index.

the granulomas contained vacuolated macrophages containing M.Ag and no epithelioid cells or MGC, associates these lesions with a deficiency in the immune response of the host. The cause of this immune deficiency in a population of TB patients and lepromatous leprosy patients has not been explained. We showed that the macrophages containing M.Ag in the multibacillary leprosy and TB lesions very strongly expressed FasL. It has been shown that antigen-presenting cells with increased expression of FasL induce apoptosis in T cells during antigen presentation, thus inducing antigen-specific T-cell tolerance [37]. The same phenomenon might lead to *M. leprae* and *M. tuberculosis* specific T-cells tolerance, ultimately causing specific immune deficiency in these individuals, as the amount of FasL in the vacuolated macrophages was very high. Recently, it has been shown that *M. tuberculosis* reactive T cells in human TB are eliminated by apoptosis [38], thus supporting our suggestion.

Epithelioid cells and MGC are characteristic features of well-formed TB granulomas, but their function is not clear. In our study in the well-formed TB granulomas, the MGC very strongly expressed FasL. The expression of Fas was either weak or absent. The epithelioid cells expressed both FasL and Fas, although the number of FasL-expressing cells was higher. Thus, the epithelioid cells and the MGC by virtue of increased expression of FasL may make these granulomas an immune-privileged site for a small number of intracellular bacilli. The Fas-expressing lymphocytes coming in contact with these FasL-expressing cells might undergo apoptosis, as is suggested for the immune-privileged status of eye [39], and testis [40]. The scarcity of lymphocytes among the epithelioid cells and the higher frequency of apoptotic cells in the epithelioid cells as compared to the lymphocytes in these TB granulomas support this hypothesis.

Fas was expressed on epithelioid cells in well-formed TB granulomas. As these cells expressed FasL as well, autocrine or juxtacrine killing of cell is expected to occur. The number of Fas-expressing cells was, however, lower than the FasL-expressing cells in the epithelioid cells in case of TB granulomas. Furthermore, mycobacteria have been shown to downregulate the Fas receptor [11]. In murine TB granulomas we have shown that the expression of Fas is weaker as compared to FasL in the infected macrophages [15]. Fas-mediated resistance to apoptosis can also be a possibility, as is found in tumour cells [41].

Calprotectin, detected by Mac387, was expressed by epithelioid cells in the TB granulomas. Interestingly calprotectin is said to possess bactericidal property [42]. Whether epithelioid cells have a protective role against *M. tuberculosis* is not known. The appearance of epithelioid cells in the lesions is shown to be associated with a decreased number of bacilli in TB [34], indicating their protective role. MGC did not express calprotectin. MGC are shown to phagocytose *M. tuberculosis*, although less efficiently than macrophages [43]. MGC are shown to express MHC class molecules and B7 antigens [44], and possibly function as atypical antigen-presenting cells. It can be speculated that MGC may represent a defined cage for antigens, able to present antigens to lymphocytes without spreading infectious agents in the environment. MGCs may also provide a sanctuary for the infectious agents by virtue of the absence of calprotectin, and the expression of FasL.

The frequency of apoptotic cells was higher in TB granulomas as compared to foreign-body granulomas. The AI was in general low ($< 1\%$) in all the granulomas we studied. The apoptotic process is, however, completed in a period of only 3 h [45]. Thus, the low number of apoptotic cells we observed is still compatible with a considerable cell death in the tissues. With TUNEL staining both apoptotic and necrotic cells were stained as chromatin is dissolved in the necrotic cells as well. However, the chromatin is fragmented early at the onset of cell death in apoptosis, whereas in necrosis the onset of the chromatin dissolution is delayed as it is a passive degenerative event [20]. The morphology of stained cells and the surrounding cells helped in the distinction between apoptosis and necrosis. The apoptotic cells were found as single cells in the midst of normal-looking cells. The necrotic cells were present as aggregates of degraded cells like the areas immediately around the necrotic centres in the TB granulomas.

In conclusion we describe a novel immune evasion mechanism for mycobacteria by virtue of increased expression of FasL in the heavily infected vacuolated macrophages in the multibacillary leprosy and TB granulomas. The well-formed TB granulomas might provide an immune privileged site for mycobacteria because of the expression of FasL possibly explaining the chronic progressive course of infection.

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REFERENCES

1 North RJ. Cell mediated Immunity and the response to infection. In: McCluskey RT, Cohen S, eds. Mechanisms of Cell-Mediated Immunity. New York: John Wiley & Sons, Inc. 1974, 185–219.

2 Chan J, Kaufmann SHE. Immune mechanisms of protection. In:

Bloom BR, ed. Tuberculosis; Pathogenesis, Protection, and Control. Washington DC: ASM Press, 1994: 389–415.

3 Placido R, Mancino G, Amendola A, *et al.* Apoptosis of human monocytes/macrophages in *Mycobacterium tuberculosis* infection. J Pathol 1997;181:31–8.

4 Klingler K, Tchou-Wong KM, Brandli O, *et al.* Effects of mycobacteria on regulation of apoptosis in mononuclear phagocytes. Infect Immun 1997;65:5272–8.

5 Keane J, Balcewicz-Sablinska MK, Remold HG, *et al.* Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. Infect Immun 1997;65:298–304.

6 Mangan DF, Welch GR, Wahl SM. Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol 1991;146:1541–6.

7 Moore KJ, Matlashewski G. Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. J Immunol 1994;152:2930–7.

8 Durrbaum-Landmann I, Gercken J, Flad HD, Ernst M. Effect of *in vitro* infection of human monocytes with low numbers of *Mycobacterium tuberculosis* bacteria on monocyte apoptosis. Infect Immun 1996;64:5384–9.

9 Munn DH, Beall AC, Song D, Wrenn RW, Throckmorton DC. Activation-induced apoptosis in human macrophages: developmental regulation of a novel cell death pathway by macrophage colony-stimulating factor and interferon gamma. J Exp Med 1995;181:127–36.

10 Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J Exp Med 1994;180:1499–509.

11 Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, Meylan PR. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. J Immunol 1998;160:5448–54.

12 Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. J Immunol 1998;161:2636–41.

13 Ottenhoff TH, Mutis T. Role of cytotoxic cells in the protective immunity against and immunopathology of intracellular infections. Eur J Clin Invest 1995;25:371–7.

14 Dockrell DH, Badley AD, Villacian JS, *et al.* The expression of Fas Ligand by macrophages and its upregulation by human immunodeficiency virus infection. J Clin Invest 1998;101:2394–405.

15 Mustafa T, Phyu S, Nilsen R, Bjune G, Jonsson R. Increased expression of Fas ligand on *Mycobacterium tuberculosis* infected macrophages: A potential novel mechanism of immune evasion by Mycobacterium tuberculosis? Inflammation 1999;23:507–21.

16 Wiker HG, Harboe M, Bennedsen J, Closs O. The antigens of *Mycobacterium tuberculosis*, H37Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis*, BCG. Scand J Immunol 1988;27:223–39.

17 Mshana RN, Humber DP, Harboe M, Belehu A. Demonstration of mycobacterial antigens in nerve biopsies from leprosy patients using peroxidase-antiperoxidase immunoenzyme technique. Clin Immunol Immunopathol 1983;29:359–68.

18 Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature 1994;372:100–3.

19 Ridley DS, Ridley MJ. Rationale for the histological spectrum of tuberculosis. A basis for classification. Pathology 1987;19:186–92.

20 Kressel M, Groscurth P. Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. Cell Tissue Res 1994;278:549–56.

21 Tanner JE, Alfieri C. Epstein-Barr virus induces Fas (CD95) in T cells and Fas ligand in B cells leading to T-cell apoptosis. Blood 1999;94:3439–47.

22 Ohshima K, Suzumiya J, Sugihara M, Nagafuchi S, Ohga S, Kikuchi M. CD95 (Fas) ligand expression of Epstein-Barr virus (EBV)-infected lymphocytes: a possible mechanism of immune evasion in chronic active EBV infection. Pathol Int 1999;49:9–13.

23 Badley AD, McElhinny JA, Leibson PJ, Lynch DH, Alderson MR, Paya CV. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. J Virol 1996;70:199–206.

24 Strand S, Hofmann WJ, Hug H, *et al.* Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells – a mechanism of immune evasion? Nat Med 1996;2:1361–6.

25 Roger PM, Bermudez LE. Infection of mice with *Mycobacterium avium* primes CD8+ lymphocytes for apoptosis upon exposure to macrophages. Clin Immunol 2001;99:378–86.

26 Kiener PA, Davis PM, Rankin BM, *et al.* Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. J Immunol 1997;159:1594–8.

27 Mariani SM, Matiba B, Baumler C, Krammer PH. Regulation of cell surface APO-1/Fas (CD95) ligand expression by metalloproteases. Eur J Immunol 1995;25:2303–7.

28 Kayagaki N, Kawasaki A, Ebata T, *et al.* Metalloproteinase-mediated release of human Fas ligand. J Exp Med 1995;182:1777–83.

29 Schneider P, Holler N, Bodmer JL, *et al.* Conversion of membrane-bound Fas (CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med 1998;187:1205–13.

30 Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. J Exp Med 1997;186:2045–50.

31 Tanaka M, Itai T, Adachi M, Nagata S. Downregulation of Fas ligand by shedding. Nat Med 1998;4:31–6.

32 Tanaka M, Suda T, Haze K, *et al.* Fas ligand in human serum. Nat Med 1996;2:317–22.

33 Hahne M, Rimoldi D, Schroter M, *et al.* Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape. Science 1996;274:1363–6.

34 Muller H, Kruger S. Immunohistochemical analysis of cell composition and in situ cytokine expression in HIV- and non-HIV-associated tuberculous lymphadenitis. Immunobiology 1994;191:354–68.

35 Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis 1966;34:255–73.

36 Boros DL. Granulomatous inflammations. Prog Allergy 1978;24:183–267.

37 Zhang HG, Su X, Liu D, *et al.* Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. J Immunol 1999;162:1423–30.

38 Hirsch CS, Toossi Z, Vanham G, *et al.* Apoptosis and T cell hyporesponsiveness in pulmonary tuberculosis. J Infect Dis 1999;179:945–53.

39 Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 1995;270:1189–92.
40 Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. Nature 1995;377:630–2.
41 Ungefroren H, Voss M, Bernstorff WV, Schmid A, Kremer B, Kalthoff H. Immunological escape mechanisms in pancreatic carcinoma. Ann N Y Acad Sci 1999;880:243–51.
42 Steinbakk M, Naess-Andresen CF, Lingaas E, Dale I, Brandtzaeg P, Fagerhol MK. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 1990;336:763–5.
43 Mariano M, Spector WG. The formation and properties of macrophage polykaryons (inflammatory giant cells). J Pathol 1974;113:1–19.
44 Burgio VL, Fais S, Boirivant M, Perrone A, Pallone F. Peripheral monocyte and naive T-cell recruitment and activation in Crohn's disease. Gastroenterology 1995;109:1029–38.
45 Bursch W, Kleine L, Tenniswood M. The biochemistry of cell death by apoptosis. Biochem Cell Biol 1990;68:1071–4.