Study of the Mechansims of Killing of Mycobacterium Bovis BCG by Apoptosis in J774 Murine Macrophages

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Abstract

Objective: To determine the relationship between the mechanism of apoptosis and intracellular killing of Mycobacterium bovis BCG. Apoptosis, “programmed cell death” - a physiologically beneficial and distinct form of cell death.
Setting: In vitro study was carried out in murine macrophage cell line J774 that was infected with Mycobacterium bovis BCG in different set of conditions. Percentage of surviving BCG and apoptotic cells was determined.
Methods: IFN-g and/or LPS-activated and non-activated J774 mouse macrophage cells were infected with BCG in a ratio of 1:5. The morphology of the host cells was studied after 4 hours, 24 hours and 48 hours of infection in cytospins stained with Jenner-Giemsa. Surviving bacteria were counted by incorporation of radiolabelled-uridine after cell lysis.
Results: Both in the activated and non-activated J774 cultures some cells undergo apoptosis. ln cells activated with IFN-g or LPS without BCG, less than 10% of cells were found to be apoptotic. More apoptosis was seen when LPSactivated cells were infected with BCG. In the cells activated with IFN-g or LPS-activated cells the percentage of apoptotic cells was much higher than in non-activated cells or cells activated with either WJF -g or LPS alone. After 24 hours culture, without BCG, about 15% of the cells were found to be apoptotic and with BCG infection this increased to 23% ($p<0.001$). The level further increased after 48 hours of infection. BCG growth inhibition was observed in both non-activated J774 cells and cells activated with LPS, INF -g or both and was sustained to 48 hours of co-culture.
Conclusion: It is evident that BCG-infected J774 cells undergo apoptosis in the presence of a high concentration of RNI and/or ROl. During this process the cells shrink considerably in volume with the removal of water that may concentrate toxic products in the cell. The increased concentration of toxic species and the disorganisation of the phagocytic vacuoles may account for the enhanced stasis and/or death of the intracellular micro-organisms. We conclude that host cell apoptosis may arrest the growth and account for the death of the intracellular mycobacterial pathogen (JPMA 49:273, 1999).

Introduction

Apoptosis is a widespread and morphologically distinct process of cell death.- it is essential for proper embryonic development and for the regulation of many rapidly grown cells in the body[1,2] and also for :the crrect functioning of the immune system[3]. Cytotoxic T-cell or natural cell kill virally infected or tumour cell by apoptosis[4].
Several laboratories have reported multiple killing mechanisms present in the same cells[5-9]. TNF and related cytotoxins provide important links between the apoptotic pathway which causes DNA degradation and perform which is able to cause membrane damage. Binding of TNF, or of antibodies directed against TNF-specific receptors, are able to cause apoptosis[10,11]. Other receptors which would engage some “apoptosin ligand” on the target cell have been postulated. These however remain to be identified.

Antimicrobial effects of RN!s have also been demonstrated in murine and human macrophages[12,15]. Activated J774 cells produced superoxide and NO in response to BCG. Co-cultures of BCG and J774 cells was found to induce RNI response in the murine macrophages. Activated J774 cells produced superoxide and NO. BCG growth inhibition was observed in both non-activated J774 cells and cells activated with LPS, INF-g or both and was sustained to 48 hours of co-culture. Addition of anti-SOD induces BCG growth inhibition of >50% in the activated host cells[16]. Nitric oxide has been shown to be involved in apoptosis in murine macrophages. Sarih et al[17] have shown that nitric oxide synthase induces macrophage death of apoptosis. Albina et al.[18] have also demonstrated the NO-dependent death of murine macrophages activated in vitro with INF-g and LPS mediated through apoptosis.
During apoptosis, some nucleases cleave chromatin between nucleosomes, so that the DNA of apoptotic cells becomes a series of fragments, all integer size multiples of the 180-200 base pair length associated with a single nucleosome. This series of fragments constitutes the characteristic 'ladder' on agarose gel electrophoresis of the DNA of apoptic cells.

Materials and Methods

BCG stock culture (Statens serum institut, Copenhagen, Denmark) was thawed, sonicated for 3 minutes to disrupt the clumps and adjusted to the required concentration in RPM!.
The J774 murine macrophages were cultured in Dulbecco's Modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco BRL). The J774 mouse macrophage cell line was activated before infection by culture for 48 hours with recombinant mouse g-interferon (Genzyme) (500 Units/mI), or 12-18 hours of culture with I mg/mI of LPS (Sigma), or 500 Units/mI of mouse g-interferon ± I mg/mi o.fLPS. Non-activated cells were also studied.
AGtivated or non-activated cells were mixed with BCG in a ratio of 1:5 (5 x 10 5 : 2.5 x 106/ well of 24 well plates) in, culture medium containing 10% (v/v) fetal calf serum in the presence or absence of DEAE-purified anti-SOD at a concentration of 5 mg/mI. Experiments were conducted in triplicate in 24 well plates (ICN Flow). After 4 hours, 24 hours and 48 hours incubation the cells were removed for microscopic examination. Cells were collected for morphological studies to identify apoptosis.
Microscopy: Cells were harvested by gentle scraping from the culture wells using a sterile rubber policeman. An even layer of single cells was prepared by cytospinning. 100 ml of the cell suspension was transferred to a cytocentrifuge and centrifugation was carried out at 1000 rpm (350g) for 5 minutes. Slides were air dried and fixed with methanol for 5 minutes. 1/3 diluted Jenner stain with the Jenner-Giemsa buffer was poured over the slides and left for 5 minutes and then washed off with distilled water. 1/10 diluted Giemsa stain in Jenner-Giemsa buffer was poured over the slides for 10 minutes and then washed offwith distilled water. Slides were dried completely and mounted in Depex mounting medium.
Morphologic changes associated with apoptosis include nuclear and cytoplasmic condensation, with the persistence of a continuous plasma membrane. Slides were examined under high power and then tinder an oil immersion lens for counting the apoptotic cells. Three hundred cells were counted and the percent of apoptotic cells determined.
BCG growth inhibition: Experiment was performed in 96 well plates to study the growth inhibition of BCG using the above system. After 4 hours of incubation extracellular bacteria were removed. Plates were incubated for 24 and 48 hours. After 24 and 48 hours of incubation BCG harvested from macrophages were counted by incorporation of radiolabelled uridine as described by Fazal et al.[19]. Briefly, after lysing the infected host cells with 4% saponin, 100 ml of 7H9 Middlebrook broth, supplemented with 10% Middlebrook ADC enrichment (Difco) was added. After 2 hour incubation, 10 ml containing lmCi of (5,6-3H)-uridine, specific activity 38 Ci/mmole (ICN Flow), was added to each well. The plates were incubated at 37°C for 2 days to allow incorporation of the uridine into the

replicating bacteria. The bacterial culture was then harvested onto fibre filter paper (printed format A, 1205-402, Pharmacia) using an automated cell harvester (Skatron Comb. CH, Finland). The filters were air dried and 9 ml of scmtillator fluid (Beta plate scint. LKB) was added before counting in a liquid scintillation counter (Beta plate LKB) for 60 seconds.

Results

Activated and non-activated 774 cells were mixed with BCG and the morphology of the host cells was studied after 4 hour, 24 hour and 48 hours of infection by cytospinning and Jenner-Giemsa's staining. Three hundred cells per sample were counted and the percent of apoptotic cells determined using the known morphology of cells undergoing apoptosis.

Morphologically cells undergoing apoptosis show a typical nuclear and cytoplasmic condensation within an intact cytoplasmic membrane (Figure),

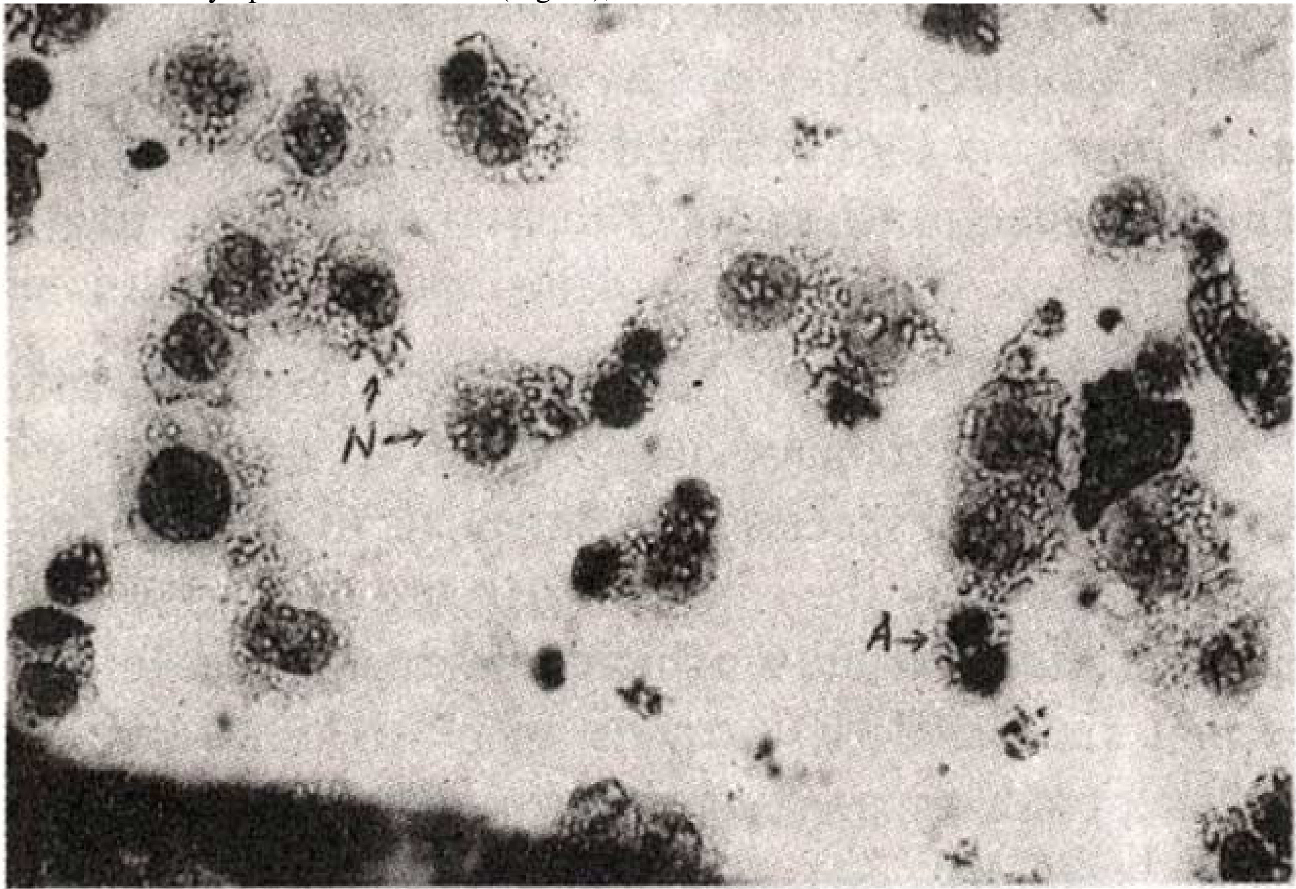

Figure. Photomicrograph (magnification x 400) of a cytospin preparation of J774 murine macrophages after 48 hours of activation with IFN-g plus LPS. The cells labelled N shows normal morphology and A indicates an apoptotic cell.

whereas those undergoing necrosis show organelle swelling, early cell membrane disruption and subsequent disintegration.

Microscopic examination show that within both the activated and non-activated J774 murine macrophage cultures some cells undergo apoptosis (Table).

Table. Percentage of apoptotic cells* and BCG growth inhibition in J774 murine macrophages cultured with or without BCG in the absence or presence of anti-SOD monoclonal Antibody.**

J774 cells	Time in hours	No treatment * / **	BCG alone * / **	Anti-SOD alone * / **	BCG+Anti-SOD * / **
IFN-γ	4	2 / -	2 / -	3 / -	3 / -
	24	5 / -	5 / 10	8 / -	7 / 45
	48	7 / -	5 / 55	8 / -	10 / 80
IFN-γ+LPS	4	12 / -	18 / -	10 / -	15 / -
	24	10 / -	15 / 38	15 / -	23 / 75
	48	17 / -	25 / 80	19 / -	30 / 95
LPS	4	3 / -	2 / -	2 / -	8 / -
	24	6 / -	4 /30	7 / -	11 / 50
	48	10 / -	12 / 42	10 / -	15 /85
Non-activated	4	2 / -	3 / -	3 / -	2 / -
	24	3 / -	3 / 8	2 / -	4 / 20
	48	2 / -	4 / 20	2 / -	1 / 25

* = % of apoptotic cells ** = % of BCG growth inhibition SOD = Superoxide dismutase

However without activation the proportion of apoptotic cells was much lower than in the activated cells. In IFN-g or LPS activated cells without BCG, less than 10% of cells were found to be apoptotic. This population increased in LPS-activated cells, when these were infected with BCG.
In the doubly activated cells the percentage of apoptotic cells was much higher than in non-activated cells or cells activated with IFN-g or LPS alone. At 4 hours of infection the apoptotic cells were in a range of 9-18%. After 24 hours of culture, without BCG, about 15% cells were found to be apoptotic and with BCG infection this was 23%. The level further increased after 48 hours of infection (Table). A significant difference (p In the doubly activated cells, a significant difference ($p<0.001$) was noted between 4 hours and 24 hours of culture, but no significant difference was seen between 24 and 48 hours. Although a comparatively higher level of apoptosis was noted in BCG-infected cells in the presence of anti-SOD m-Ab (Table), the effect of adding antibody did not give a statistically significant result.
Growth of BCG was inhibited in J774 cells cultured for up to 48 hours after activation with IFN-g, LPS or both. Little growth of BCG was also inhibited in non-activated cells. Culture with anti-SOD antibodies reduced the number of viable organisms harvested from J774 after 24 hours by about a half-log of CFU/well. This applied to both non-activated cells and those activated by g-interferon and/or LPS. We have also observed that g-interferon- plus LPSactivated cells were more active in inhibiting the growth of BCG than g-interferon activated cells.

Discussion

The development of multicellular organism involves a delicate balance among the processes of proliferation, differentiation and death. Naturally occurring cell death aids tissue remodelling, eliminates redundant cell populations and provides structural elements such as hair and skin. In the nervous system, selective cell death contribute to formation and organisation of spinal cord, retina etc[20,21].
Apoptotic cells do not induce an inflammatory reaction and they are the target of immediate phagocytosis either by macrophages or by neighbouring cells. Macrophages or adjacent viable cells recognise the cells that are undergoing apoptosis and remove efficiently these unwanted cells from tissues without release of potential toxic cell contents which might have otherwise damaged

neighbouring cells and elicited an inflammatory response.
Sarih et al[17] and Albina et al[18] have demonstrated an NO-dependent death of murine macrophages activated in vitro with IFN-g or LPS, which is mediated through apoptosis. We have shown that activated J774 murine macrophages produce high levels of NO_2- after 24 hours and this rises over 48 hours. BCG was found to induce a characteristics NO_2- response in activated J774 cells. Furthermore addition of anti-SOD m-Ab to BCG infected cells was found to induce a marked rise in NO_2- synthesis after 24 hours of culture[16]. In addition high levels of NO2 in IFN-g plus-LPS activated cells was associated with about 50% macrophage death over 48 hours of culture[16]. Albina et al[22] had previously shown that murine macrophages, the effector cells for NO-mediated cytotoxicity, themselves become the target for NO toxicity and die in culture when activated to express NO. The following experiments were formed to examine this phenomenon in further detail as it is clearly relevant in considering the relationship between intracellular bacterial survival and host cell response.
We have observed NO-dependent death of murine macrophages activated in vitro with IFN-g and LPS which are mediated through apoptosis. Apoptosis was observed in both the activated and non-activated cells. However the level of apoptosis was different in these groups (Table). A significantly higher level (p<0.00l) was noted in the doubly-activated cells. We have also observed that ginterferon- plus LPS-activated cells were more active in inhibiting the growth of BCG than g-interferon activated cells.
It has been shown that IFN-g and LPS activation of J774 murine macrophages induces high levels of ROI and RNI. These toxic species have potentials to cause cellular damage. Probably the extent of damage of macrophages by ROl and RNI is too high, the repair mechanisms become futile and NO activate apoptosis. ROl and RN! and their products are known to cause DNA damage. It has been suggested that it may be too dangerous for lymphocytes to attempt repair of certain kinds of damage, as faulty repair could lead to autoimmunity or leukaemia; instead they activate their apoptotic mechanism. Interestingly BCG infection in the presence of anti-SOD rn-Ab in the doubly-activated cells further enhanced (by about 5%) the level of apoptosis. As we have seen that BCG infection in the presence of anti-SOD enhances both the ROI and RN! response in J774, so this difference in level of apoptosis could be due to a higher level of RN! induced in the presence of anti-SOD.
Apoptosis is observed in tuberculous granulomata. Recently Klinger et al[23] concluded that apoptosis of mononuclear phagocytes induced by M. tuberculosis occurs in vivo and in vitro model of mycobacterial infection. The induction of apoptosis in alveolar macrophages by M. tuberculosis niay play a role in the macrophage-pathogen interaction of tuberculosis in vivo[24]. Many animal viruses are known to induce an apoptotic response in infected cells, the significance of virus-indcued apoptosis is not yet clear, It is possible that under certain stress conditions the cells before surrendering to pathogen take last step to switch on the suicide mechanism that may lead to death of both host and pathogen. This supports the proposition that there is a suicide program in many vertebrate cells which can be activated when the cells's death is desirable for the good of the rest of the cell community[25]. In BCG infection J774 murine macrophages underwent apoptosis in the presence of high concentration of RN! or ROL Probably in order to make a final attempt to kill micro-organism, nature has given this mammalian cell type macrophages, a unique property to undergo apoptosis. During this process the cells considerably shrink in volume with the removal of water that may concentrate toxic products in the cells. The increased concentration of toxic species could lead to death of the intracellular micro-organisms as well.
Zychlinsky et al[26] have reported that virulent strains of Shigella dysentriae induces apoptosis in J774 murine macrophages. It is also possible that mycobacteria also induce apoptosis within infected macrophages to aid the spread of the organism from one macrophage to another without re-entering the hostile extra-cellular environment. It is also possible that some T cells induce apoptosis, instead of lysis to prevent inflammatory responses produced by lysis of mycobacterial infected macrophages.

Recently it has been shown that cellular sensitivity or resistance to TNF, which regulates levels of intracellular ROI[27] and induces apoptosis, is correlated with decreased or increased levels of SOD[28]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism and many inhibitors of apoptosis have antioxidant activities[27,29,30]. Buttke and Sandstrom[31] have suggested that eukaryotic cells may benefit from this perilous existence by invoking oxidative stress as a common mediator of apoptosis.

We have studied the apoptosis mechanism in J774 and found that enhanced levels of RNI coincides with apoptosis in these macrophages. The percentage of apoptotic cells was highest in doubly-activated BCGinfected cells in the presence of anti-SOD monoclonal antibody. Anti-SOD was found to enhance the NO production in the BCG infected cells, so this high percentage of apoptosis could be due to a high level of NO. This observation agrees with others[17,18] who deduced that IFN-g and LPS can induce apoptosis through the nitric oxide pathway.

In conclusion both ROt and RNI or their products may be involved in the process of both the induction of apoptosis and intracellular killing ofmicro-organisms. It is difficult to determine what benefit cells may get from apoptosis in infection. Apoptosis may follow bacterial damage due to high levels of ROl and RNI and then to be phogocytosed by fresh macrophages.

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