Induction of IL-1 β , IL-6, TNF- α , GM-CSF and G-CSF in human macrophages by smooth transparent and smooth opaque colonial variants of *Mycobacterium avium*

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Summary. Both smooth transparent (SmT) and smooth domed-opaque (SmD) colonial variants were obtained from a strain of *Mycobacterium avium* isolated from a patient with AIDS. The two variants showed similar biochemical characteristics but SmT bacteria proliferated better than SmD bacteria inside human macrophages and were much less capable than the SmD variant of inducing the release of IL-1 β , IL-6, TNF- α , GM-CSF and G-CSF, after incubation for either 3 or 6 days. As cytokines are important extracellular signals for immune cells, the lack of induction observed in SmT-infected macrophages may be one of the pathogenic mechanisms of *M. avium*.

Introduction

Mycobacterium avium-intracellulare complex (MAC) is a large group of acid-fast bacilli of environmental origin that yield three colonial variants when grown in agar: smooth transparent (SmT), smooth domed-opaque (SmD) and rough (Rg) variants.^{1,2} The SmT variant was found to be more resistant to drugs and chemicals^{3,4} and more pathogenic for chickens and mice than the SmD variant,^{2,5} suggesting some relationship between colonial morphology with virulence. After transfer of the bacteria from host tissues to in-vitro culture conditions, transition from the SmT to the SmD colony type was observed.¹ MAC strains with all three types of colonial variants could be isolated from human immunodeficiency virus (HIV)-negative patients with chronic lung infections, with the SmD and SmT forms predominating.⁶ In contrast, blood isolates of MAC from patients with AIDS gave only SmT colonies on primary cultures;⁷ in these patients, MAC disseminated throughout the body within macrophages with an adverse effect on survival.^{8–10}

The mechanisms of MAC pathogenicity are poorly understood despite the abnormalities known to be induced in macrophage functions or T cell-macrophage interactions.^{11–14} It has been shown^{15–17} that MAC infection of human monocytes or macrophages was able to induce the release of IL-1 β , IL-1 α , IL-6, GM-CSF and TNF- α .

However, these studies were done by infecting cells

with bacteria harvested from Löwenstein-Jensen agar slants or Middlebrook 7H9 broth, which are known to give a selective advantage to the SmD variant.⁷ Michellini-Norris *et al.*¹⁸ reported that SmT and SmD variants induced a differential release of IL-1 α , IL-1 β and IL-6 from normal human monocytes and suggested that disturbances of cytokine production could contribute to MAC survival within cells, and then establish the infection.

The aim of this study was to examine the production of cytokines from SmD- or SmT-infected monocyte-derived human macrophages, for a more complete evaluation of the release of these immunoregulatory proteins following MAC stimulation.

Materials and methods

Bacteria

Mycobacterium avium strain 485 (serovar 21), was isolated from the blood of a patient with AIDS. It was identified by the Gen Probe Rapid Diagnostic System (Gen Probe, San Diego, CA, USA) and serotyped by Dr A. Tsang at the National Jewish Center for Immunology and Respiratory Medicine of Denver, CO, USA.¹⁹

Isolation of colonial variants

The bacteria were grown on Middlebrook 7H10 Agar (Difco) for 1–2 weeks at 37°C in a humidified atmosphere of air with CO₂ 5%. In the first cultures, the plates contained essentially SmT colonies with

only a few SmD colonial variants. The SmT variants were suspended in Middlebrook 7H9 Broth (Difco) and stored at -40°C until use. One opaque colony was streaked on to a 7H10 plate and incubated for 2 weeks; after re-isolation of the opaque colonies, cultures containing only SmD variants were obtained.

The colonies were observed by both a stereo-microscope (Wild, Heerbrugg, Switzerland) and an inverted microscope (Leitz, Wetzlar, Germany). Photographs were taken on Ilford films.

Identification procedures

SmT and SmD colonial variants were stained by the Kinyoun method²⁰ and identified by standard biochemical tests²⁰ including growth on medium containing NaCl 5% and MacConkey agar without crystal violet, nitrate reduction, ariylsulphatase and pyrazinamidase production, heat-stable (68°C) catalase production, tellurite reduction, Tween 80 hydrolysis, urease and niacin (INH test strips, Difco) production.

Preparation of human macrophages

Leucocyte buffy coats obtained from healthy donors were diluted 1:1.5 in sodium chloride 0.9% and peripheral blood mononuclear cells (PBMC) were separated over Ficoll-Hypaque (Histopaque 1077; Sigma).²¹ PBMC were washed twice and resuspended in RPMI 1640 medium containing 25 mM HEPES (Gibco Laboratories, Grand Island, NY, USA) supplemented with 2 mM L-glutamine and heat-inactivated (56°C , 30 min) fetal calf serum 10% (complete medium). The cell suspension was adjusted to $2 \times 10^7/\text{ml}$ and distributed in 0.5-ml volumes in 24-well tissue-culture plates (Costar 3524, Cambridge, MA, USA). After incubation for 2 h at 37°C in humidified air with CO_2 5%, the non-adherent cells were aspirated and the monocytes ($2\text{--}4 \times 10^5/\text{well}$) were washed vigorously twice with warmed complete medium without antibiotics and counted by the method of Nakagawara and Nathan.²² After incubation for 4 days > 95% of the cells were phagocytes, as demonstrated by their ability to ingest neutral red. Viability was determined by trypan blue dye exclusion test.

Establishment of infection

Bacteria were prepared by suspending either SmT or SmD colonies from 7H10 agar plates in complete medium up to an optical density of 0.2 at 500 nm. To decrease bacterial clumping, suspensions were sonicated for 10 s (Ultrasonicator Soniprep 150; MSE; 8 μm amplitude); after this treatment, the number of cfu/ml increased from 2.2×10^7 to 1.1×10^8 for SmD and from 2.2×10^8 to 5.6×10^8 for SmT. To infect macrophages, monolayers were exposed to either SmT or SmD bacteria at a ratio of 100:1 (MAC/ml: macrophages/well) for 2 h at 37°C in humidified air

with CO_2 5%. Infected monolayers were washed twice to remove extracellular bacteria, incubated in complete medium and used for the determination of cytokine levels and viable counts.

Cytokine determination

Culture fluids were harvested on days 3 and 6, centrifuged at 14000 *g* for 30 min to eliminate bacteria, and frozen in small volumes at -40°C until assayed. Cytokine levels were measured by an ELISA assay (Quantikine; R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Viable counts

After harvesting culture fluids for cytokine determination, the infected monolayers were lysed to determine the number of viable bacteria/ml of macrophage lysate. Distilled water (0.5 ml) was added to each well and, after incubation for 10 min at room temperature, 0.5 ml of a lysing solution (110 ml of 7H9 medium, 40 ml of sodium dodecylsulphate 0.25% in 0.1 M sodium phosphate buffer, pH 7), was added to each well for a further 10 min. The wells were scraped vigorously with a pipette and 0.5 ml of bovine serum albumin 20% in sterile water was added. For colony counting, macrophage lysates were serially 10-fold diluted and 0.5 ml volumes of each dilution were plated in triplicate on 7H10 agar. The numbers of cfu were counted after incubation of the plates for 10–14 days at 37°C in humidified air with CO_2 5%.

Results

Microbiological properties of the SmT and SmD variants

The colonial morphology of both SmT and SmD variants is shown in fig. 1. The SmT variant (panels A and B) produced smooth translucent colonies with a central whitish dot and a diameter of *c.* 0.3–0.5 mm, after incubation for 1 week. The SmD variant (panel C) showed opaque and dome-shaped colonies of *c.* 1–2 mm diameter after incubation for 2 weeks.

Both variants gave negative results in tests for growth in medium containing NaCl 5% and on MacConkey agar, Tween 80, urease and niacin tests, and positive results in pyrazinamidase and tellurite tests. Furthermore, the SmD variant showed slightly positive results in ariylsulphatase and nitrate tests but a negative reaction for heat-stable catalase; the SmT variant gave negative results in the first two tests and positive results for heat-stable catalase.

Kinyoun's staining showed that SmT bacteria were, as expected, small red coccobacilli; in contrast, bacteria from SmD colonies were, to a great extent, non-acid fast and appeared as small blue bacteria. The results were the same, regardless of the solid medium for growth (Middlebrook 7H10 or Löwenstein-Jensen).

Fig. 1. Photomicrographs of *M. avium* colonial variants growing on Middlebrook 7H10 agar. A, SmT colonies observed by inverted microscope; bar = 0.1 mm. B, SmT colony observed by stereomicroscope; bar = 0.1 mm. C, SmD colony observed by stereomicroscope; bar = 0.2 mm.

Intramacrophagic growth of *M. avium* colonial variants

To investigate the ability of SmT and SmD variants to grow inside human macrophages, monolayers prepared from three different blood donors were infected with the mycobacterial cell variants. Both infected and non-infected macrophages retained > 95% viability during the whole period of incubation.

A representative experiment illustrating the growth of SmT and SmD bacteria inside macrophages is shown in fig. 2. The mean intracellular bacterial density on day 0 was 1.8×10^5 cfu/ml of macrophage lysate for the SmT and 1.2×10^5 cfu/ml for the SmD variant. As each well contained *c.* 2×10^5 macrophages, it can be assumed that each macrophage contained *c.* 0.9 SmT or 0.6 SmD bacteria, respectively. The number of intracellular SmT bacteria increased *c.* seven times on day 3 and 14 times on day 6. As for the SmD variant, *c.* 70% of the cells in the initial inoculum were killed on day 3 and a slight regrowth on day 6 was observed. Of the survivors and regrown bacteria on day 6, *c.* 4.5% reverted to the SmT variant.

Cytokine production

The time course of the release into culture fluid of IL-1 β , IL-6, TNF- α , GM-CSF and G-CSF by uninfected or SmT- or SmD-infected macrophages is reported in fig. 3, panels a–e, respectively; the data shown refer to the experiment shown in fig. 2.

The SmD variant strongly stimulated the release of all the cytokines tested with increasing production to day 6, except for GM-CSF, which was abundant on

Fig. 2. Growth of SmT (○) and SmD (△) colonial variants of *M. avium* in human macrophages after incubation for 0, 3 and 6 days. Viable counts (cfu/ml) of macrophage lysates for triplicate wells are shown. (Bar = SD).

day 3 but decreased on day 6. In contrast, the SmT variant did not induce the release of the cytokines tested to any appreciable extent.

Control uninfected cells did not release any cytokine. Two other experiments with macrophages from

Fig. 3. Release of a, IL-1 β ; b, IL-6; c, TNF- α ; d, GM-CSF; e, G-CSF into culture fluid by uninfected (□) and SmT-infected (○) or SmD-infected (△) human macrophages. Values are expressed as picograms/10⁵ macrophages. Data reported refer to the experiment shown in fig. 2. (Bar = SD).

different subjects produced viable counts and cytokine concentrations that were essentially the same as those shown in figs. 2 and 3, respectively (data not shown).

Discussion

This report focuses on the differential ability of SmT and SmD variants of a strain of *M. avium* to grow inside human macrophages and to cause the release of cytokines into the medium.

SmT and SmD variants showed similar biochemical characteristics but SmD bacteria were mostly non acid-fast. This unexpected finding might be related to some differences between the cell wall of the two variants, as supported by the fact that SmT bacteria contain a thick polysaccharide-rich outer layer which is absent or discontinuous in the SmD cells.^{1,23}

When human macrophages were infected with the SmD variant, most bacteria were killed rapidly by the phagocytes with abundant release of cytokines. In contrast, the SmT variant multiplied rapidly inside macrophages without any appreciable induction of cytokines. Thus, our data confirm the findings of Michelini-Norris *et al.*¹⁸ concerning IL-1 β and IL-6 in

monocytes, but also extend them to other cytokines, such as TNF- α , GM-CSF and G-CSF not previously studied.

As cytokines are important activation signals for immune cells, the lack of induction observed in SmT-infected macrophages may explain one of the possible pathogenic mechanisms for *M. avium*. This suppressive activity could affect the initial immune response to the micro-organism *in vivo*, thus enabling the infection to become established. The suppression involves some of the most important macrophage-derived cytokines modulating inflammation and the acute phase response (IL-1 β , IL-6, TNF- α), as well as proliferation and differentiation of haemopoietic progenitor cells (GM-CSF and G-CSF).²⁴ These findings are consistent with the reported isolation of SmT rather than SmD variants from the blood of AIDS patients. They are also in keeping with observations *in vivo* that, despite the extraordinary load of mycobacteria found in macrophages of MAC-infected AIDS patients, inflammatory responses are limited and granulomas are poorly formed.⁸ This trend seems to be different from that observed in tuberculosis in which cytokines are produced *in vitro* in response to mycobacterial products,²⁵⁻²⁶ or *in vivo* during active illness,²⁷⁻²⁸ and participate in granuloma formation in mice.²⁹

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