Fungicidal activity of Candida albicans-induced murine lymphokine-activated killer cells against C. albicans hyphae in vitro

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Multiple intraperitoneal injections of inactivated Candida albicans cells resulted in the generation of cytotoxic peritoneal cells with phenotypical and functional properties similar to in vitro-generated lymphokine-activated killer (LAK) cells. Using an in vitro [3H]glucose uptake assay, C. albicans-induced LAK-like (CA-LAK) cells exhibited high levels of anti-hyphal activity, the effects being effector to target cell (E:T) ratio- and time-dependent. Maximal levels of anti-C. albicans activity (approximately 60%) were observed after 4 h and at E:T ≥ 300:1. Similar patterns of anti-C. albicans activity were exerted by in vivo-activated natural killer (NK) cells, in vitro interleukin-2- (IL-2) generated LAK cells and polymorphonuclear cells. The anti-hyphal activity of CA-LAK cells was enriched by separation on a Percoll gradient, F2 and F3 fractions retaining most of the activity. Experiments using immunodepressed animals demonstrated that the in vivo lethality of the C. albicans hyphal form is significantly affected by in vitro pre-exposure to CA-LAK cells. While control mice receiving C. albicans alone had a median survival time of 2 d, mice receiving C. albicans pre-exposed to CA-LAK cells (E:T = 300:1) had a median survival time of 15 d. Overall, the susceptibility of the C. albicans hyphal form to CA-LAK cells suggests that C. albicans-induced effectors might play a significant role as a second-line defence mechanism against the C. albicans hyphal form.

Introduction

Natural killer (NK) cells and lymphokine-activated killer (LAK) cells play an important role in host defence mechanisms against tumours and infectious agents (Cerottini & Brunner, 1974; Haskill et al., 1975; Herberman et al., 1975; Marconi et al., 1985; Abo et al., 1986; Scaringi et al., 1988). We have recently demonstrated that murine LAK-like cells can be generated in vivo by multiple injections of inactivated Candida albicans (CA) (Scaringi et al., 1989, 1990). These CA-induced effector cells (CA-LAK cells), which closely resemble the in vitro interleukin-2- (IL-2) induced LAK cells according to morphological and phenotypical criteria (Le For et al., 1988; Hasui et al., 1989), also possess wide-spectrum cytotoxicity for both NK-sensitive and NK-resistant tumour targets. These, as well as previous data (Saito et al., 1986), imply that microbial elements can play a role, with a mechanism yet to be identified, as inducers of cytotoxic cells and, in particular, of LAK-like cells in vivo. Whether such effectors can, in turn, exert antimicrobial activity remains a matter of debate. Initial evidence has been provided by Beno & Mathews (1990), which demonstrates that in vitro IL-2-induced cytotoxic lymph node cells mediate in vitro growth inhibition of the C. albicans hyphal form, thus suggesting a potential anti-fungal role for non-phagocytic lymphoid cells. In an attempt to gain more insights on this issue, we questioned whether CA-LAK cells generated in vivo by multiple C. albicans administrations (Scaringi et al., 1990) could also exert anti-hyphal activity in vitro. Using a [3H]glucose uptake assay (Scaringi et al., 1991), we show that CA-LAK cells are active against the C. albicans hyphal form. Such activity is effector to target cell (E:T) ratio- and time-dependent. Interestingly, the CA-LAK-mediated anti-C. albicans activity is comparable to that detected for IL-2-induced LAK cells, activated NK cells or polymorphonuclear (PMN) cells.

Abbreviations: CA, inactivated C. albicans; E:T ratio, effector to target cell ratio; (r)IL-2, (human recombinant) interleukin-2; LAK cells, lymphokine-activated killer cells; LGL, large granular lymphocyte; MST, median survival time; NK cells, natural killer cells; PMN, polymorphonuclear.
Our results suggest the possibility that non-phagocytic effector cells, i.e. LAK and activated NK cells, might play a direct role as anti-C. albicans host defence elements during fungal infection.

Methods

Candida albicans. C. albicans (strain CA-6), isolated from a clinical specimen (Marconi et al., 1976), was grown at 28 °C with mild agitation in low-glucose Winge medium as described by Mattia & Cassone (1979). Under these conditions, cultures yielded about 3 x 10⁸ cells ml⁻¹ and the micro-organisms grew as an essentially pure yeast-form population. Cells were harvested from Winge medium, washed twice in saline and suspended in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum and 0.1% (w/v) gentamicin sulphate (referred to as complete medium). All reagents were purchased from Flow Laboratories. The suspension was purchased from Flow Laboratories. The suspension was dispensed in flat-bottomed 96-well microtitre plates (400 µl per well) and incubated at 37 °C in 5% (v/v) CO₂. The occurrence of a dimorphic transition in low-glucose Winge medium as described by Mattia et al. (Scaringi et al., 1989) after 3 h incubation. These hyphal forms were used both in the in vitro and in vivo assays.

Mice. Hybrid (Balb/c x C57BL/6) F1 (CD2F1, H-2b/H-2k) mice of both sexes were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy).

Human recombinant IL-2. Human recombinant IL-2 (rIL-2) was obtained from Boehringer Mannheim.

Drugs. Cyclophosphamide (Endoxan, Asta Werke, Germany) was dissolved in a sterile 0.85% (w/v) NaCl solution immediately before use and injected intraperitoneally in a volume of 0.1 ml per 10 g body weight. Polynosinic-polycytidylic acid (pol(y)C) was purchased from Sigma.

Effector cells. In vitro-generated CA-LAK cells were obtained from 6-8-week old mice injected (2 x 10⁷ cells per mouse) with five doses of C. albicans inactivated by overnight exposure to 40 mg sodium methiobol 1⁻¹, as described previously (Scaringi et al., 1989). Peritoneal exudate cells were harvested and fractionated by passage through a nylon-wool column, as detailed below. The non-adherent fraction, previously identified as CA-LAK cells (Scaringi et al., 1990), was used in the present study as the effector cell population.

In vitro IL-2-induced LAK cells were obtained from freshly isolated splenocytes (Hasui et al., 1989). Briefly, 5 x 10⁷ cells per ml were cultured in complete medium supplemented with 5 x 10⁻⁵ M-2-mercaptoethanol in 24-well tissue culture plates (Falcon Plastics) in the presence of 100 U ml⁻¹ IL-2 at 37 °C in a humidified environment of 5% CO₂ in air. After 3 d incubation, the cells were harvested, washed twice in complete medium and used for the cytotoxicity assay as described below. The viability of cultured cells was approximately 75%, as determined by trypan blue dye exclusion.

Activated NK cells were obtained from spleens of 12-week old mice injected with pol(y)C (5 mg kg⁻¹) 3 d earlier. Spleen cell suspensions were obtained by standard methods and fractionated by passage through a nylon-wool column, as detailed below. The non-adherent fraction was identified as activated NK cells and these were used as effector cells in the present study.

PMN cells were obtained from the peritoneal cavities of thioglycolate-treated mice (1 ml 10%, i.v., broth) 18 h earlier. About 95% of the cells were classified as PMN cells by morphological criteria.

Cell fractionation procedures. Nylon-wool column. Effector cells were passed through a nylon-fibre column, as described by Julius et al. (1973). Briefly, sterile nylon columns were rinsed with 20 ml RPMI 1640 medium supplemented with 5% (v/v) foetal calf serum and put in a CO₂ incubator at 37 °C at least 1 h before loading the cells. Then, 10⁶ cells in 2 ml complete medium were added and re-incubated for 45 min at 37 °C. The columns were then eluted with warm medium, the first 25 ml of each effluent was collected in a conical tube and cells were pelleted by centrifugation.

Discontinuous Percoll density gradient. Nylon-wool non-adherent cells were layered onto a six-step discontinuous density gradient of Percoll (Pharmacia) according to the modified method of Timonen & Saksela (1980). The tubes were then spun at 550 g for 30 min. Cells from the six layers were collected from the top with a syringe and washed twice in serum-free RPMI 1640 medium before being used in the assays.

Cell morphology was evaluated in May–Gr¨unwald–Giemsa-stained cytocentrifuge slides. Two-hundred cells were scored on each slide.

Cytotoxicity assay. The microassay against tumour cells has been described previously (Marconi et al., 1985). The tumour cell lines (YAC-1 and EL-4), cultured in vitro in complete medium, were used as targets. Various numbers of effector cells (0.1 ml per well) were incubated in U-shaped 96-well microtitre plates with 3² Chromium prelabelled tumour cells (5 x 10³ per well in 0.1 ml). After 4 h incubation, the plates were centrifuged at 800 g for 10 min, and the radioactivity in 0.1 ml of the supernatants was measured with a γ-scintillation counter. All groups were tested in quadruplicate. The baseline ³⁵Cr release value was determined using an autologous control with equal numbers of unlabelled target cells in the place of effector cells. The percentage anti-tumour activity was calculated as follows:

\[
\text{Percentage activity} = \left( \frac{\text{test c.p.m.} - \text{autologous c.p.m.}}{\text{total c.p.m. incorporated}} \right) \times 100
\]

where test c.p.m. is the mean c.p.m. released in the presence of effector cells, and total c.p.m. incorporated is the mean c.p.m. incorporated in 5 x 10⁵ tumour target cells.

Assay for anti-C. albicans activity. Various numbers of effector cells (0.1 ml per well) were added to C. albicans hyphae (4 x 10⁵ per well, in 0.1 ml). The cells were obtained as detailed above. The plates were then incubated for 4 h at 37 °C in 5% CO₂. The medium in the wells was discarded by inverting the microplate or by gentle pipetting (virtually no C. albicans cell loss was observed). [³H]glucose ([5,6-³H]glucose, New England Nuclear) was then added (5 µCi ml⁻¹ (185 kBq ml⁻¹) in sterile water, 50 µl per well) according to the method of Djeu et al. (1986). After an additional 3 h incubation at 37 °C, the radioactivity incorporated by the residual C. albicans was harvested by adding 5% (w/v) sodium hydrochloride (50 µl per well) and then processed through a MASH harvester onto glass-fibre filters and read in a β-scintillation counter. The mean of the triplicate cultures was determined and the percentage anti-C. albicans activity was calculated as follows:

\[
\text{Percentage activity} = \left( \frac{\text{c.p.m. C. albicans with effectors}}{\text{c.p.m. C. albicans alone}} \right) \times 100
\]

Effector cells alone did not incorporate significant levels of radioactivity (approximately 0.2–1% of the c.p.m. incorporated by C. albicans alone).

Evaluation of pathogenicity of C. albicans incubated in vitro with or without CA-LAK cells. C. albicans hyphal forms, seeded into six-well plates (10⁴ per well), were incubated with or without CA-LAK cells at various E:T ratios. After 4 h, the content of each well was detached by a scraper, harvested with 1 ml syringe (24 guage needle) and injected intravenously into immunodepressed mice (150 mg cyclophosphamide kg⁻¹ on day 3). There was no significant clumping in any of the experimental groups and virtually all the material was recovered from
the wells (data not shown). Ten mice per group were injected and the median survival times (MSTs) recorded.

Statistical analysis. Statistical analysis was determined by the Student's $t$ test or the Mann-Whitney U-test. All experiments were repeated three to five times. Mean values of all experiments or representative results are shown in Tables 1-4 and Fig. 1.

Results

Recently, we have demonstrated that in vivo injection of inactivated C. albicans induces, in the peritoneal cavity of mice, an anti-tumour cytotoxic population, closely resembling in vitro IL-2-induced LAK cells (Scaringi et al., 1989, 1990). Thus, it was of interest to determine whether such C. albicans-induced LAK-like cells also exhibited anti-fungal properties. For this purpose, we used a recently established microassay (Scaringi et al., 1991) which allows measuring of cell-mediated anti-hyphal activity in vitro. In particular, CA-LAK effector cells were incubated with C. albicans at an E:T of 300:1 for different times and then the anti-hyphal activity was assessed as $[^3H]$glucose uptake by residual hyphae. We found that CA-LAK cells were active against C. albicans. A minimum of 2 h interaction between CA-LAK cells and C. albicans was needed to obtain significant levels of anti-hyphal activity (Table 1). Maximal levels were observed at 4–6 h, whereas after 24 h incubation, the anti-C. albicans effects partially decreased. When peritoneal exudate cells from placebo-treated mice were fractionated through a nylon-wool column and co-incubated with C. albicans for 4 h, no significant levels of anti-hyphal activity were observed (data not shown). These results indicated that C. albicans-induced peritoneal cells possessed anti-fungal activity, measurable in vitro against the C. albicans hyphal form.

With the purpose of establishing whether other non-phagocytic cytotoxic cells could also exert anti-hyphal activity, comparative studies were performed in which CA-LAK cells, in vitro IL-2-induced LAK cells and in vivo-activated NK cells were assessed in the $[^3H]$glucose uptake assay. PMN cells, known to be active against hyphal forms of C. albicans in other experimental systems (Baccarini et al., 1985; Diamond et al., 1978),

Table 1. Time-dependent anti-C. albicans activity of CA-LAK cells

<table>
<thead>
<tr>
<th>Time of co-incubation (h)</th>
<th>Mean c.p.m.* ± SD</th>
<th>Mean anti-C. albicans activity† (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. albicans alone</td>
<td>C. albicans + effector cells</td>
</tr>
<tr>
<td>1</td>
<td>7757 ± 648</td>
<td>7058 ± 458</td>
</tr>
<tr>
<td>2</td>
<td>10350 ± 1044</td>
<td>7762 ± 823</td>
</tr>
<tr>
<td>3</td>
<td>13625 ± 660</td>
<td>7221 ± 678</td>
</tr>
<tr>
<td>4</td>
<td>14250 ± 1050</td>
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</tr>
<tr>
<td>6</td>
<td>17350 ± 1261</td>
<td>6940 ± 974</td>
</tr>
<tr>
<td>24</td>
<td>45501 ± 3141</td>
<td>32765 ± 2027</td>
</tr>
</tbody>
</table>

* The values are the mean c.p.m. per well of five independent experiments ± SD.
† The results are elaborated and expressed as percentage anti-C. albicans activity as described in Methods.
were also included. We found that IL-2-induced and CA-LAK cells had superimposable patterns of activity. Significant levels of anti-hyphal activity were observed at E:T ratios of 50:1, whereas maximal levels were reached at E:T ratios ≥ 300:1 (Fig. 1a, b); NK cells were also active to a similar extent (Fig. 1c). PMN cells exerted the highest levels of anti-C. albicans activity (Fig. 1d), which, however, decreased to undetectable levels at E:T ratios < 25:1 (data not shown).

We have previously demonstrated that the anti-tumour effector cells within the CA-LAK population are non-adherent, unaffected by carbonyl-iron and magnet treatment and possess large granular lymphocyte (LGL) morphology, in that they can be enriched by fractionation on a Percoll gradient (Scaringi et al., 1989, 1990). In order to determine the distribution of the anti-C. albicans effector cells along a Percoll gradient, the CA-LAK cell population was fractionated onto a six-step Percoll gradient and then tested against C. albicans and tumour targets. As expected (Scaringi et al., 1989, 1990), F2 and F3 retained most of the anti-tumour activities which were associated with the highest content of cells with LGL morphology (Table 2). Interestingly, these fractions also retained most of the anti-hyphal activity. F4, which showed a significantly reduced percentage of cells with LGL morphology as compared to F2 and F3 (20.4% versus, 80.1 or 65.3%, respectively), also exhibited lower levels of anti-C. albicans activity. F5, composed primarily of small lymphocytes, did not exert significant antimicrobial activity.

In further experiments, F2 and F3 were depleted of phagocytic cells by carbonyl-iron and magnet treatment prior to being assessed for anti-C. albicans activity. Both fractions retained all their anti-hyphal activity, regardless of the fact that they were or were not depleted for phagocytic cells (Table 3). These results indicated that the CA-LAK-mediated anti-C. albicans activity was independent of the presence of contaminating phagocytic cells.

We then investigated whether the CA-LAK cell-mediated anti-C. albicans effects observed in vitro could be associated with reduced pathogenicity in vivo. Thus, C. albicans hyphal forms were incubated with or without CA-LAK cells at various E:T ratios and then injected into immunodepressed mice. Mice receiving C. albicans pre-exposed in vitro to CA-LAK cells showed a significant enhancement of the MST with respect to control mice receiving C. albicans alone (Table 4). The CA-LAK-mediated effects on C. albicans pathogenicity were dependent upon the E:T ratios employed and
Table 4. Anti-hyphal activity of CA-LAK cells: correlation between in vitro anti-C. albicans effects and in vivo pathogenicity

<table>
<thead>
<tr>
<th>In vitro incubation:*</th>
<th>In vitro assay:</th>
<th>In vivo assay:</th>
</tr>
</thead>
<tbody>
<tr>
<td>E:T ratio</td>
<td>Anti-C. albicans activity (%)</td>
<td>Group</td>
</tr>
<tr>
<td>0:1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>50:1</td>
<td>21.0</td>
<td>2</td>
</tr>
<tr>
<td>100:1</td>
<td>29.7</td>
<td>3</td>
</tr>
<tr>
<td>300:1</td>
<td>57.4</td>
<td>4</td>
</tr>
</tbody>
</table>

* CA-LAK cells and/or C. albicans were incubated for 4 h at the indicated E:T ratios.
† Significantly different from controls (P < 0.01).

Discussion

Evidence exists that natural defence mechanisms are crucial in reducing the pathogenic burden of C. albicans both in normal and immunodepressed hosts (reviewed in Odds, 1988). Because of their phagocytic properties, PMN cells and macrophages represent a useful first line defence barrier against C. albicans infections, as documented by numerous studies on the C. albicans yeast-form (Baccarini et al., 1983, 1986; Bistoni et al., 1983; Decker et al., 1986; Djeu et al., 1986a; Fromling & Shadomy, 1986; Djeu & Blanchard, 1987; Leijh et al., 1977; Blasi et al., 1990, 1991; Kolotila & Diamond, 1990; Vecchiarelli et al., 1991). However, the dimorphic transition from the yeast to the hyphal form, enables C. albicans to avoid phagocytic clearance and invade tissues, where virtually only hyphae are found (reviewed in Odds, 1988). In vitro studies demonstrate that PMN cells are potent anti-hyphal effector cells, exhibiting mechanisms other than ingestion and intracellular killing against this fungal form (Diamond et al., 1978; Baccarini et al., 1985).

Our data demonstrate that a non-phagocytic effector cell, generated in vitro by administration of C. albicans (Scaringi et al., 1989, 1990), is active against the C. albicans hyphal form in vitro. Unlike peritoneal cells from placebo-treated mice, the C. albicans-induced cell population constitutively exhibits anti-hyphal activity that is strictly dependent upon the E:T ratios employed and is accomplished within a few hours. Thus, similar to anti-tumour activity, CA-LAK-mediated anti-C. albicans activity is a short-term event, which most likely requires an effector to target cell interaction. In fact, CA-LAK cell free supernatants fail to exert any effect on the C. albicans hyphal form (data not shown).

Percoll gradient fractionation of the CA-LAK population demonstrates that F2 and F3 fractions, which retain most of the CA-LAK anti-tumour cytotoxicity, also exhibit the highest anti-hyphal activity. In contrast, F5, which mainly contains small lymphocytes with a few PMN cells (5.3%), has no antimicrobial properties. This implies that anti-C. albicans activity is expressed by a specific lymphoid subpopulation, i.e. that with LGL morphology, which is likely to be the same subpopulation that mediates the anti-tumour effects. The possibility that contaminating cells, such as PMN cells, may play any direct and/or indirect role within the CA-LAK effector population, although not fully excluded, remains unlikely since F5, which contains 5.3% of contaminating PMN cells has no anti-hyphal activity, and F2 and F3, once depleted of phagocytic cells, still retain 100% of their anti-C. albicans activity. Thus, we feel confident in concluding that a C. albicans-induced non-phagocytic effector cell is active against hyphal forms of C. albicans. Meanwhile, it remains uncertain whether the anti-C. albicans activity of the CA-LAK population is due to an NK cell, to a cell with the LAK-like phenotype or rather to the co-operation between the two. Although not addressed here, our comparative studies demonstrate that NK cells as well as in vitro-generated LAK cells possess anti-hyphal activities and their activities closely resemble that of CA-LAK cells in terms of E:T ratio and time-dependency. Overall, these results indicate that three different non-phagocytic effector cells, obtained either in vivo or in vitro, are all capable of mediating anti-hyphal activity in vitro. This finding extends further a recent contribution on the issue of lymphoid cells as anti-C. albicans effectors (Beno & Mathews 1990). These authors have shown that significant anti-hyphal activity is exerted by lymph node cells pre-exposed in vitro to IL-2 and conclude that the active effector cell is functionally and phenotypically similar to an activated NK cell. While previous reports (Diamond et al., 1978, 1981; Baccarini et al., 1985; Scaringi et al., 1991) have shown that hyphae can be damaged by PMN cells and monocytes, our data together with those of Beno & Mathews (1990) indicate that non-phagocytic effector cells can also exert anti-hyphal activity.

Experiments with immunodepressed animals demonstrate that the pathogenic burden of C. albicans is highly impaired following in vitro incubation with CA-LAK cells. The effect is dependent upon the E:T ratios used in the in vitro co-incubation and correlates with the anti-hyphal activity as evaluated in vitro by the [3H]glucose uptake assay. In our opinion, the in vivo experiments provide additional evidence on the anti-hyphal role of
CA-LAK cells, at least during an in vitro contact. At the moment, we cannot exclude the possibility that, by the use of secretory products, CA-LAK cells, may activate other populations in vitro, which in turn contribute to contain the infection.

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