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Activation of cytokine genes during primary and anamnestic immune response to inactivated *C. albicans*

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SUMMARY

Recent evidence suggests that after repeated stimulations with inactivated *C. albicans* (CA) cells, CD2F1 mice respond with a cytokine pattern typical of T-helper 1 (Th1) subset development. The purpose of this study was to analyse the sequence of immunological events which, soon after priming mice with CA, lead to the development of primary and anamnestic response. A comprehensive kinetics analysis of cytokine mRNA expression was performed by Northern blot assay, in peritoneal exudate cells (PEC), at different phases of immune response to CA: after priming (one i.p. injection of 2×10^7 CA cells/mouse), during development of the primary immune response (five progressive CA i.p. injections over a 2-week period) and in the anamnestic response (CA booster 30 days after the primary response). *In vitro* assays were performed 2 and 24 hr after every CA stimulation. The response to CA priming was characterized by an early and high expression of interleukin-2 (IL-2) and IL-1β mRNAs. At 24 hr, IL-2 mRNA was still at a high level, while IL-1β had greatly decreased. A weak expression of IL-10 was only induced at 2 hr, whereas IL-12 p40 subunit, interferon-γ (IFN-γ), IL-4 and IL-5 mRNAs were undetectable. In this phase no *in vitro* proliferative response of PEC to CA was observed, whereas a significant natural killer (NK) activity was induced. From the second CA injection, the IFN-γ mRNA was already induced at 2 hr. Its expression level increased progressively with the number of CA injections persisting up to 24 hr after the fifth stimulation. A progressive increase of IL-2 mRNA expression was also induced whereas IL-1β and IL-10 mRNAs were always transiently expressed at 2 hr at levels similar to those observed after the priming. IL-12 p40 subunit, IL-4 and IL-5 mRNAs were never detectable. The expression of this selected cytokine pattern typical of Th1 response was correlated with the development of CA-specific T lymphocytes as confirmed by the *in vitro* proliferative response of CA-5d-induced PEC to CA. NK activity also increased progressively with the number of CA injections and after the fifth stimulation lymphokine-activated killer (LAK) activity was also induced. The anamnestic response to CA was characterized by a very quick induction of high levels of IL-2, IFN-γ and IL-1β mRNAs. IL-2 and IFN-γ mRNAs remained high up to 24 hr while IL-1β mRNA decreased strongly. A weak, transient expression of IL-10 mRNA was induced at 2 hr whereas the IL-12 p40 subunit, IL-4 and IL-5 mRNAs were not detectable. The presence of CA-specific memory lymphocytes was confirmed by the *in vitro* specific proliferative response of PEC to CA. CA booster caused also a very rapid and high level of NK/LAK activation. In conclusion, these results indicate that CA is able to progressively trigger differentiation of the Th1 subset which develops in the absence of IL-12, and that Th memory cells retain the same selected Th1 cytokine profile developed in the primary immune response.

INTRODUCTION

Several infectious disease models have clearly demonstrated that protection or progression of disease depends mainly upon which T-helper (Th) subset, Th1 or Th2, is preferentially activated during the infection.[1–4] Differentiation of the Th subset is often apparent in the early stages of infection[5] and among factors influencing development of the appropriate

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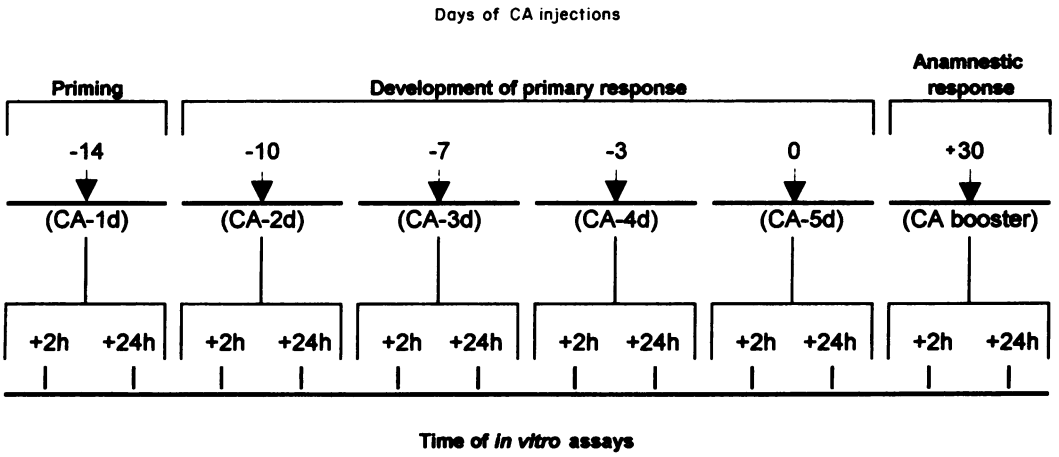

Figure 1. Experimental plan. CD2F1 mice were stimulated with different numbers of CA i.p. injections (2×10^7 cells/mouse). *In vitro* assays were performed at the times reported after every CA injection.

immune response, cytokines, to which naive T cells are exposed soon after antigen stimulation, are the most important.[6,7] This indicates that effective therapies for infectious diseases might include administration of recombinant cytokines able to promote the protective Th subset development[8–10] and neutralization, by monoclonal antibodies, of cytokines which instead promote the non-protective Th subset.[2,11] An additional important strategy to potentiate resistance to infection is the development of vaccine using inactivated microorganisms or their immunological active components when these can induce the development of the Th subset associated with protective immunity.

Studies on *C. albicans* experimental infection in mice have evidenced the central role of polymorphonuclear cells[12] and macrophages[13] as effectors of resistance to infection and the strict correlation between Th1 subset differentiation and development of protective immunity.[3,14] Therefore, the prevention of candidiasis, particularly important in situations associated with weakness in host defences, could be achieved by stimulating the immune system to develop a Th1 response and, more important, by inducing a state of long-lasting specific memory.

Previous results have demonstrated that inactivated *C. albicans* (CA) has many effects on the natural immune system, the most important of which are the induction of natural killer/lymphokine-activated killer (NK/LAK) cells[15–17] and activation of macrophages in the peritoneal cavity of mice.[18] Besides, repeated CA stimulations induce a cytokine pattern typical of Th1 subset development.[19] These results led to further studies to determine the mechanisms that drive the immune system towards the development of Th1 response to CA and the nature of the anamnestic response. Advances in this field could be very important for a possible application of CA in vaccination.

To gain further knowledge on this topic, we analysed the cytokine pattern expressed in the peritoneal exudate cells (PEC) after CA priming, during the development of the primary immune response and in the anamnestic response. The murine peritoneal cavity was used as a study model because it is a useful anatomical site for analysing immune event kinetics.[17,20,21]

Our results show that the development of the Th1 subset in response to CA occurs by interleukin-12 (IL-12) independent mechanisms and this selected Th1 response persists as a long-lasting immunological memory.

MATERIALS AND METHODS

Animals

Hybrid (BALB/c Cr × DBA/2 Cr)F1 (CD2F1, H-2^d/H-2^d) and inbred C57BL/6 mice, of both sexes, 7–8 weeks old, were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy).

Microorganisms

The C. albicans (strain CA-6), used throughout this study, was isolated from a clinical specimen[22] and identified by established taxonomic criteria.[23,24] Cultivation, inactivation and lyophilization of the microorganisms were performed as previously described.[15]

The CA suspension (1×10^8 cells/ml), prepared immediately before use in sterile 0·85% NaCl solution, was injected intraperitoneally (i.p.) in a total volume of 0·2 ml/mouse according to the experimental plan reported in Fig. 1 or diluted at the appropriate concentration for *in vitro* proliferation assay.

Group B streptococcus Type Ia (strain 090, GBS-Ia) was kindly provided by Dr J. Jelinkova (Institute of Hygiene ans Epidemiology, Prague, Czech Republic). The microorganisms were cultured overnight in Todd–Hewitt broth (THB) and the number was estimated turbidimetrically at 540 nm in a Beckman DU-68 spectrophotometer (Beckman Instruments, Fullerton, CA). After washing and heat inactivation (30 min at 60°) GBS was diluted in RPMI-1640 medium (Flow Laboratories, McLean, VA) for *in vitro* proliferation assay.

Tumour cell lines

Cell lines, YAC-1 (a tissue culture cell line of a Moloney-induced lymphoma of A/Sn origin), P815 (a methylcholanthrene-induced mastocytoma of DBA/2 origin) and EL-4 (a benzopyrene-induced lymphoma of C57BL/6 origin) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter referred to as complete medium). All

reagents were purchased from Flow Laboratories (McLean, VA). YAC-1 and P815 tumour cells were used as targets in the ^{51}Cr release assay.

EL-4 tumour cells were stimulated for 20 hr with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Company, St. Louis, MO) before RNA harvesting for Northern blot analysis.[25]

Cell populations

Peritoneal exudate cells (PEC) were harvested by peritoneal washing with 10 ml cold RPMI-1640 medium containing 5 U/ml heparin. Spleen cells (SC) were recovered by standard procedures. Cells were washed three times in cold complete medium and the viability was determined by trypan blue dye exclusion.

Cell fractionation procedures

Plastic adherence. PEC were plated (2×10^6 cells/ml) in tissue culture dishes and incubated for 1 hr at 37° in a CO_2 incubator. Then, non-adherent cells were recovered by washing with warm complete medium.

Passage through nylon wool column Effector cells were passed through a nylon-wool fibre column, as previously described.[16] Briefly, sterile nylon columns were rinsed with complete medium and warmed in a CO_2 incubator at 37° at least 1 hr before loading the cells. Then, 10^8 cells in 1 ml of complete medium were added and reincubated for an additional 45 min.

The columns were then eluted with warm complete medium and the first 25 ml of each effluent collected in a conical tube.

^{51}Cr release assay

The microassay against tumour cells has been previously described.[15] Target cells were labelled with 200 μCi of $Na^{51}CrO_4$ (Du Pont de Nemours, MI, Italy). The cells were then washed twice with complete medium and added at 5×10^3 cells/well to various numbers of effector cells in round-bottomed microtitre plates (Greiner Labortechnik, Nürtingen, Germany). After 4 hr incubation at 37° in 5% CO_2, the plates were centrifuged at 800 ***g*** for 10 min, and the radioactivity in 0·1 ml of the supernatant measured by a γ-scintillation counter. All groups were tested in quadruplicate. The baseline ^{51}Cr release value was determined using an autologous control instead of effector cells. The percentage of specific lysis was calculated as:

$$\% \text{ cytotoxicity} = \frac{\text{test c.p.m.} - \text{autologous c.p.m.}}{\text{total c.p.m. incorporated}/2} \times 100$$

Test c.p.m. is the mean c.p.m. released in the presence of effector cells. Total c.p.m. incorporated is the mean c.p.m. incorporated in 5×10^3 tumour target cells.

Northern blot analysis

Total cellular RNA was isolated from PEC, SC and EL-4 cells by using a single-step phenol/chloroform extraction procedure.[26] Poly $(A)^+$ mRNA was enriched by oligo-dT-affinity chromatography according to Sambrook *et al.*[27] Total RNA (20 μg) and Poly $(A)^+$ mRNA (8 μg) were denatured by heating for 10 min at 65° in a solution of 20 mM MOPS buffer, pH 7·0, containing 6·5% formaldehyde, 50% formamide, 5 mM sodium acetate and 1 mM ethylene diamine tetraacetic acid (EDTA). Separation of mRNA by electrophoresis was performed using a 1% agarose/2·2 M formaldehyde gel with 80 volts of applied current. RNA was then transferred to a nitrocellulose filter membrane (Schleicher & Schuell, Dassel, Germany) by Northern capillary blotting using 10× SSC (1× SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7·2). The filter was baked for 2 hr at 80° and then prehybridized for 4 hr at 42° in 0·1 M phosphate buffer (pH 6·8), 50% formamide, 5 × SSC, 5 × Denhardt's solution (0·1% Ficoll, 0·1% polyvinylpyrrolidone, 0·1% bovine serum albumin), 0·2% sodium dodecyl sulphate (SDS) and 100 μg/ml denatured salmon sperm DNA (Sigma Chemical, Co. St. Louis, MO). Hybridization was carried out for 18 hr in the same solution by adding a specific ^{32}P-labelled cDNA probe. The ^{32}P-labelled probe was obtained using the Nick translation kit (Boehringer Mannheim Biochemicals, Indianapolis, IN), according to the procedure suggested by the manufacturer. The specific activity of the labelled probe was $2–5 \times 10^8$ d.p.m./μg DNA. After hybridization, the filter was washed in 2× SSC, 0·1% SDS for 20 min at room temperature and then in 0·2× SSC, 0·1% SDS for 30 min at 60°. The filter was blotted dry and exposed to Kodak X-ARS film (Eastman-Kodak Company, Rochester, NY) for 24 hr at −80° in the presence of an intensifying screen.

To ensure that equal amounts of RNA were analysed, blots were stripped and reprobed with β-actin cDNA. The intensities of radiographic signals were quantified by using laser densitometry (Ultrascan XL, LKB Instruments, Houston, TX). The cDNA probes for murine IL-2, IL-4 and IL-5 were obtained from American Type Culture Collection (Rockville, MD). The IL-12 cDNA was generously supplied by Dr Giorgio Trinchieri (Winstar Institute of Anatomy and Biology, Philadelphia, PA), IL-1β cDNA was supplied by Dr L. Varesio (National Cancer Institute, Frederick, MD), IFN-γ and β-actin cDNA by Dr S. Landolfo (Institute of Microbiology, Turin, Italy), IL-2 receptor α chain (IL-2Rα) cDNA by Dr J. Miller (National Institutes of Health, Bethesda, MD) and IL-10 cDNA by Dr Manuela Baccarini (Fraunhofer Institut, Hannover, Germany).

Flow cytometric analysis

The dynamics of lymphomyeloid cell populations recruited by CA in the peritoneal cavity was evaluated in all the experimental conditions tested, by cytofluorimetric analysis of cell phenotype. T helper (Th) lymphocytes were evaluated by L3T4 antigen expression and B lymphocytes by the expression of Ly-5 antigen.

NK cells were detected by using anti-asialo GM1 antibody. To better characterize NK cells, we analysed the expression of NK cell surface antigen detected by the 3A4 monoclonal antibody (mAb) in C57BL/6 inbred mice.[28] C57BL/6 mice were also used to detect the expression of CD25 activation marker, by mAb specific to the α chain (p55) of IL-2R. Myelomonocytic cells were characterized using anti-MAC-2 (macrophages) and anti-Gr-1 (polymorphonuclear cells) antibodies.

The fluorescein-conjugated antibodies used were: rat mAb anti-L3T4, rat mAb anti-MAC-2, sheep anti-rabbit immunoglobulin G (IgG) $F(ab')_2$ (Boehringer Mannheim Biochemica, Germany), rat mAb anti-Ly-5, rat mAb RB6–8C5 (anti Gr-1, a granulocyte marker), rat mAb anti-IL-2 receptor (α chain specific), (Pharmingen, San Diego, CA), goat anti-mouse IgM (Pierce, Rockford, IL).

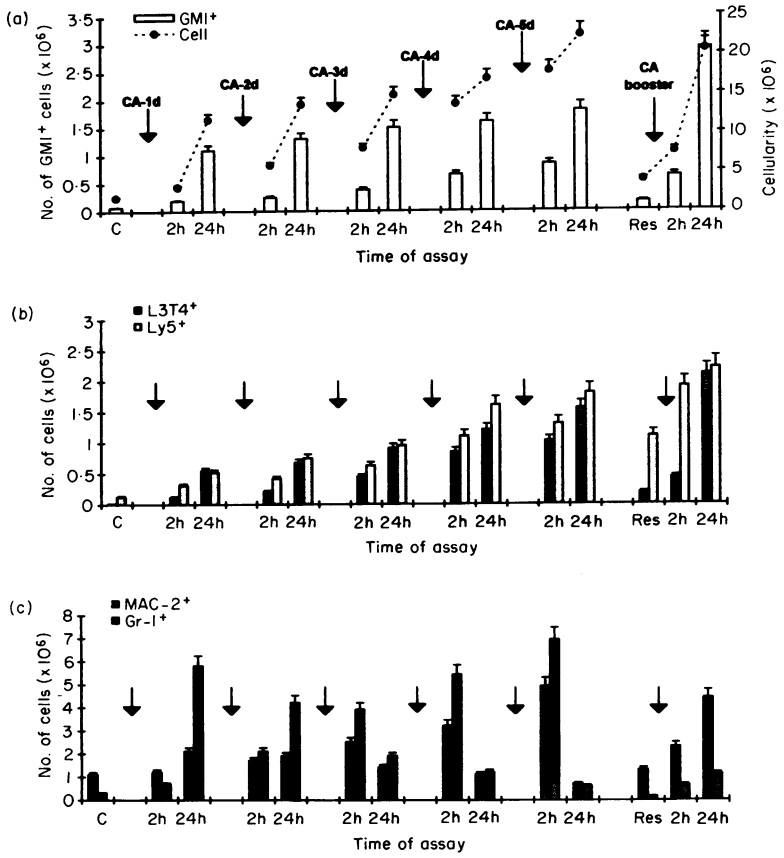

Figure 2. Phenotypic analysis of PEC at different phases of immune response to CA. PEC were recovered from untreated CD2F1 mice (C), 2 and 24 hr after every progressive CA stimulation, 30 days after CA-5d (Res) and 2 and 24 hr after CA booster. Number of positive cells at cytofluorimetric analysis. Data are presented as the mean values ± SD of four experiments. Cellularity was evaluated as number of cells recovered from the peritoneal cavity. Data are presented as the mean values of individual counts of 10 peritoneal exudates ± SD in four experiments.

The purified antibodies used were: rabbit anti-asialo GM1 serum (Wako Chemicals, GmbH, Dusseldorf, Germany), mouse mAb 3A4 (an anti-mouse NK antigen) (Pharmingen, San Diego, CA).[28]

Cells were harvested and resuspended at 1×10^6 per tube in staining buffer (phosphate-buffered saline, PBS, 0·1% sodium azide, 0·1% bovine serum albumin). After centrifugation, antiserum or fluoresceinated mAb was added to the pellet of cells which were then incubated for 30 min at 4°. The cells were then washed twice and those requiring a second step were incubated with fluorescein-conjugated sheep $F(ab')_2$ anti-rabbit IgG or goat anti-mouse IgM, for an additional 30 min at 4°. Flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACS) analyser 440 (Becton Dickinson, Mountain View, CA).

The data reported are the means of values obtained in four different experiments and expressed as absolute number.

Proliferation assay

Plastic-non-adherent PEC (5×10^6 cells/ml) were cultured in triplicate, in flat-bottomed microtitre plates (Falcon Plastics, Los Angeles, CA) in complete medium or in medium containing CA (1×10^5 cells/ml) or heat-inactivated GBS-Ia (1×10^7 microorganisms/ml). The plates were incubated for 48 hr at 37° in 5% CO_2 before adding [^{3}H]thymidine, 1 μCi/well, (NEN Research Products, Boston, MA) for an additional 18 hr. The cells were harvested on a multiple suction filtration apparatus (Multimash 2000, Dytateck, Billingshurst, UK). The ^{3}H-labelled radioactivity bound to the filter paper disc was measured by a β-scintillation counter.

Calculation of lytic units

Dose–response curves were obtained in selected experiments by plotting the percentage of specific ^{51}Cr release and the effector-to-target ratio (three to five for each curve). The best-fit curve for these functions was logarithmic (Hewlett-Packard calculator H-p 97: program standard Pac. 03–01) in agreement with previous reports.[29,30] A lytic unit (LU_{10}) was defined as the number of effector cells extrapolated from the dose–response curve required to achieve 10% specific target cell killing. The amount of LU_{10} per peritoneal exudate was calculated by dividing the total number of PEC by the number of cells corresponding to one LU_{10}.

Statistical analysis

The Northern blot analysis was repeated three times and results reported are those of a typical experiment. Cytotoxicity assay was repeated six times while cytofluorimetric analysis and

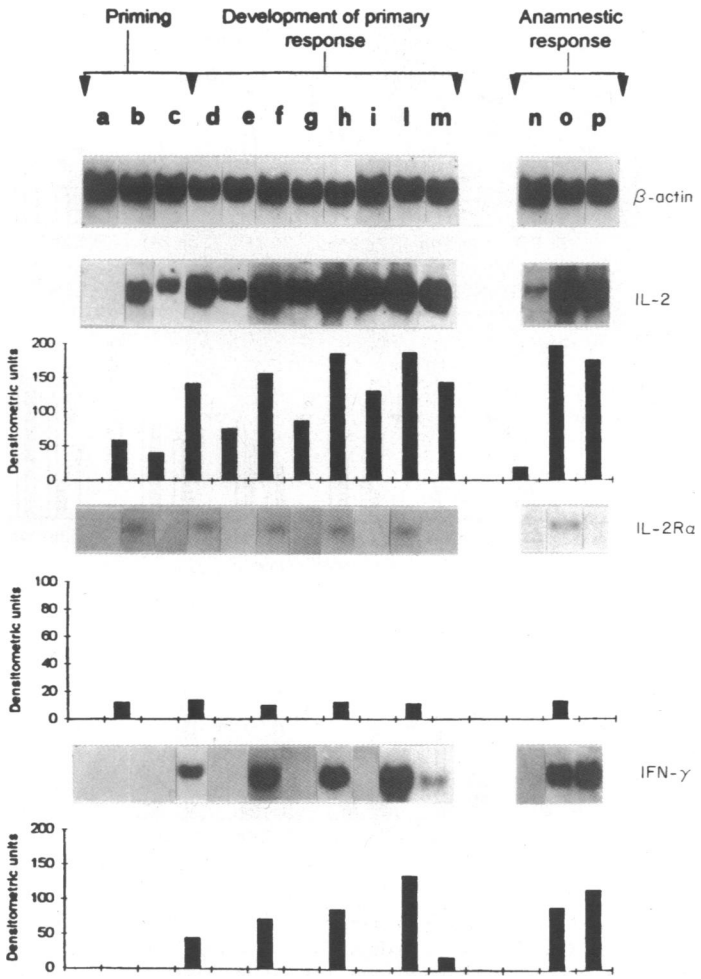

Figure 3. Kinetics of *in vivo* IL-2, IL-2Rα chain and IFN-γ mRNA expression in PEC at different phases of immune response to CA. Northern blot analysis was performed on 20 μg of total cellular RNA isolated from PEC of untreated CD2F1 mice (lane **a**) and PEC recovered at 2 and 24 hr after injection of CA-1d indicated in lanes **b** and **c**, CA-2d in lanes **d** and **e**, CA-3d in lanes **f** and **g**, CA-4d in lanes **h** and **i** and CA-5d in lanes **l** and **m**. PEC recovered 30 days after CA-5d is indicated in lane **n** and PEC recovered at 2 and 24 hr after CA booster in lanes **o** and **p**. Results of densitometry analysis are presented as a histogram after IL-2, IL-2Rα chain and IFN-γ levels were relative to β-actin levels and expressed as densitometric units. Lane designations are identical for both blots and histograms.

proliferation assay were repeated four times. The data are presented as mean values ± SD. Proliferation data were analysed by Student's *t*-test.

RESULTS

To analyse the immune mechanisms operating early, during the development of primary and in the anamnestic response, CD2F1 mice were primed with CA, progressively stimulated with repeated CA injections over a two week period and then boosted 30 days after the development of the primary immune response (Fig. 1).

Lymphomyeloid cell population recruitment

The kinetics of lymphomyeloid cell population recruitment in the peritoneal cavity was evaluated by phenotype cytofluorimetric analysis of the PEC recovered 2 and 24 hr after every CA stimulation (Fig. 1).

Figure 2a shows that CA priming induced a strong influx of immune cells in the peritoneal cavity. Cell recruitment increased progressively with the number of CA injections. Cellular response to CA booster was more rapid and greater than that induced by CA priming.

Cytofluorimetric analysis shows that asialo $GM1^+$ cells (Fig. 2a), $L3T4^+$ cells and $Ly\text{-}5^+$ cells (Fig. 2b) influxed rapidly in the peritoneal cavity after CA priming reaching at 24 hr a higher number with respect to that observed at 2 hr. The influx of these cell populations increased progressively after every CA stimulation. However, a quicker and greater recruitment was induced in response to CA booster.

After CA priming macrophages and polymorphonuclear cells (PMN) also influxed in the peritoneal cavity with the same kinetic recruitment as lymphoid cells. However from the third CA stimulation onwards, the influx kinetics of phagocytic cells changed, since the number of these cells was higher at 2 hr with respect to 24 hr. In response to CA booster, the number of phagocytic cells increased until up 24 hr and the recruitment of

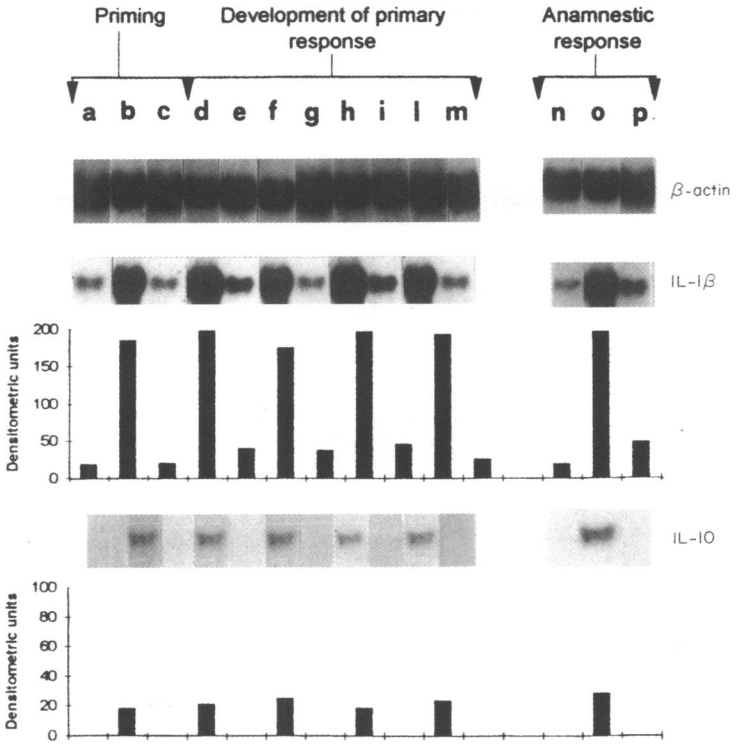

Figure 4. Kinetics of *in vivo* IL-1β and IL-10 mRNA expression in PEC at different phases of immune response to CA. Northern blot analysis was performed on 20 μg of total cellular RNA isolated from PEC of untreated CD2F1 mice (lane **a**) and PEC recovered at 2 and 24 hr after injection of CA-1d indicated in lanes **b** and **c**, CA-2d in lanes **d** and **e**, CA-3d in lanes **f** and **g**, CA-4d in lanes **h** and **i** and CA-5d in lanes **l** and **m**. PEC recovered 30 days after CA-5d is indicated in lane **n** and PEC recovered at 2 and 24 hr after CA booster in lanes **o** and **p**. Results of densitometry analysis are presented as a histogram after IL-1β and IL-10 levels were relative to β-actin levels and expressed as densitometric units. Lane designations are identical for both blots and histograms.

macrophages, but not PMN, was greater than that induced by CA priming (Fig. 2c).

Cytokine mRNA expression

The cytokine pattern was examined in the PEC recovered 2 and 24 hr after every CA stimulation (Fig. 1).

Analysis of IL-2 expression shows that the transcript, absent in peritoneal cells of control mice, was readly induced at a high level by CA priming and its expression increased progressively after every CA stimulation. Thirty days after CA-5d a low level of IL-2 mRNA was still expressed in the peritoneal resident cells but after CA booster, it increased dramatically at 2 hr and persisted at a high level until 24 hr (Fig. 3).

IL-2Rα mRNA, undetectable in unprimed mice, was expressed only 2 hr after every CA injection both during the development of the primary and in the anamnestic response (Fig. 3).

IFN-γ mRNA was undetectable in peritoneal cells both from control and CA-primed mice. A transient expression was induced for the first time 2 hr after CA-2d. The expression level of this cytokine, evaluated at 2 hr, increased progressively with the number of CA stimulations and after CA-5d persisted up to 24 hr. Thirty days later, IFN-γ mRNA was absent in peritoneal resident cells but it was strongly induced 2 hr after CA booster and its expression was still high at 24 hr (Fig. 3).

Analysis of IL-1β inflammatory cytokine shows that mRNA constitutively expressed at a low level in peritoneal cells of untreated mice, increased greatly 2 hr after every CA injection to then decrease at 24 hr both during the development of the primary and in the anamnestic response (Fig. 4).

Specific IL-10 mRNA, undetectable in peritoneal cells of control mice, showed during the development of the primary and in the anamnestic response only a transient expression 2 hr after every CA stimulation (Fig. 4).

Analysis of the IL-12 p40 subunit, IL-4 and IL-5 expression, assessed in Poly $(A)^+$ mRNA, demonstrates that these cytokines were never induced by CA (Fig. 5).

Correspondence between mRNA expression and *in vitro* cytokine production by CA-induced PEC was demonstrated in all the experimental conditions examined, by biological assays or enzyme-linked immunosorbent assay (ELISA) for IL-1β, IL-2, IL-10, IFN-γ (data not shown).

Proliferative response

Since the immune system of mice responded to progressive CA stimulations with a cytokine pattern typical of Th1 subset development, experiments were performed to determine whether the induction of this selected cytokine profile corresponded to the activation of CA-specific T cells. To this end, we examined the *in vitro* proliferative response of PEC induced 24 hr after: CA priming, CA-5d and CA booster, to the specific (CA) and non-specific (GBS-Ia) antigenic stimuli.

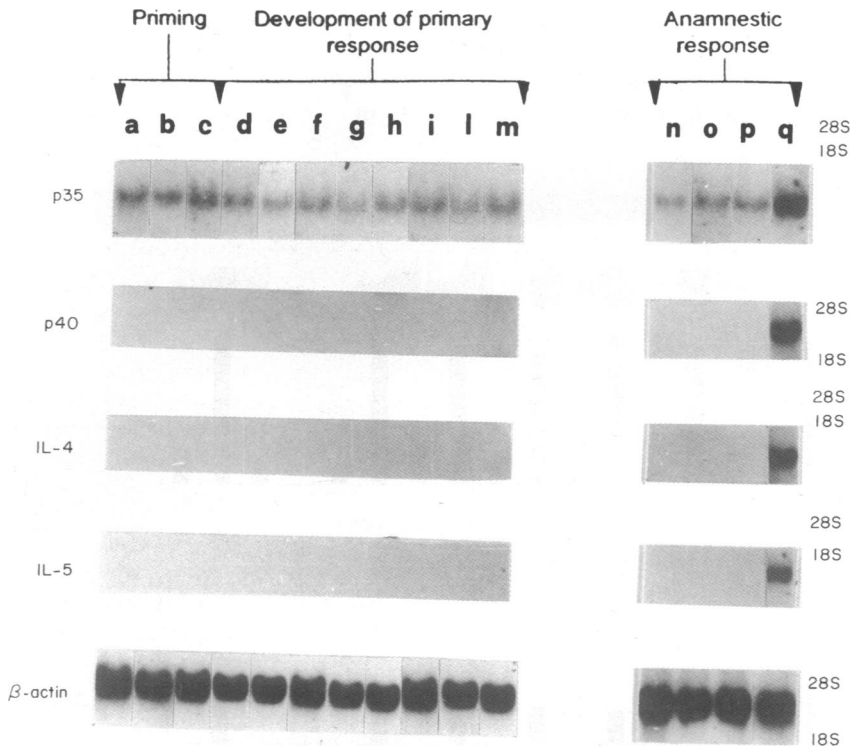

Figure 5. Analysis of *in vivo* IL-12 (p35 and p40 subunits), IL-4 and IL-5 mRNA expression in PEC at different phases of immune response to CA. Northern blot analysis was performed on 20 μg of total cellular RNA isolated from PEC of untreated CD2F1 mice (lane **a**) and PEC recovered at 2 and 24 hr after injection of CA-1d indicated in lanes **b** and **c**, CA-2d in lanes **d** and **e**, CA-3d in lanes **f** and **g**, CA-4d in lanes **h** and **i** and CA-5d in lanes **l** and **m**. PEC recovered 30 days after CA-5d is indicated in lane **n** and PEC recovered at 2 and 24 hr after CA booster in lanes **o** and **p**. Poly $(A)^+$ mRNA isolated from spleen cells of untreated mice was used as positive control for IL-12 expression (lane **q**). Poly $(A)^+$ mRNA isolated from PMA-stimulated (10 ng/ml for 20 hr) EL-4 cells were used as positive control for IL-4 and IL-5 expression (lane **q**). The same blots were stripped and reprobed with β-actin cDNA to estimate the relative amounts of Poly $(A)^+$ mRNA.

In these experiments, plastic non-adherent cells (Nad-PEC) were used since we demonstrated previously that in CA-induced PEC, adherent cells have strong anti-proliferative activity (unpublished observations).

Figure 6 shows that after CA priming Nad-PEC were unable to proliferate in response to specific and non-specific antigens. Instead, after CA-5d and CA booster, Nad-PEC showed a specific proliferative response to CA.

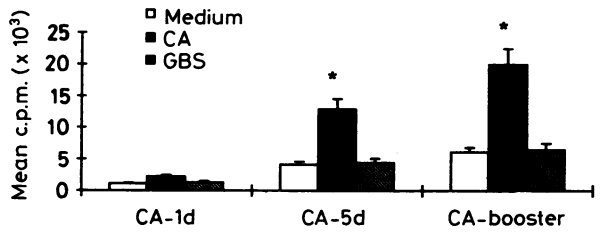

Figure 6. *In vitro* proliferative response to CA of PEC induced *in vivo* by CA at different phases of the immune response. Non-adherent cells were obtained by fractionation on plastic dishes of PEC recovered 24 hr after CA-1d, CA-5d and CA booster. Cells were incubated *in vitro* with CA (1×10^5 cells/ml) or heat-inactivated GBS-Ia (1×10^7 microorganisms/ml) for 3 days. Data are presented as the mean values ± SD of four experiments.*$P < 0.01$ (antigen-stimulated cells *versus* medium cultured cells).

NK activation

Since the expression level of IL-2 and IFN-γ mRNAs, strong inducers and potentiators of NK activity, increased progressively in the PEC with the number of CA stimulations, we determined whether this event was correlated with a progressive enhancement of NK peritoneal activity. Figure 7a shows that NK activity increased progressively with the number of CA stimulations. A significant level of LAK activity was also induced after CA-4d and CA-5d.

NK activity, which decreased to the value of untreated control mice 30 days after CA-5d, was quickly induced by CA booster at 2 hr and further increased at 24 hr. At this time a significant increase in LAK activity was also observed.

It is worth noting that in all phases of immune response to CA the NK activity peak corrisponded to when the number of asialo $GM1^+$ cells was higher. Since asialo GM1 antigen is also expressed on macrophages and activated T lymphocytes,[31] a better characterization of NK cells was performed to correlate their number with the increase of non-major histocompatibility complex (MHC)-restricted cytotoxicity. To this end, inbred C57BL/6 mice were used to analyse the expression of a NK cell surface antigen by 3A4 mAb and to detect the IL-2Rα chain activation marker.

Figure 7(b) shows that the number of 3A4+ cells and IL-$2R\alpha^+$ cells increased concomitantly with NK activity, reaching

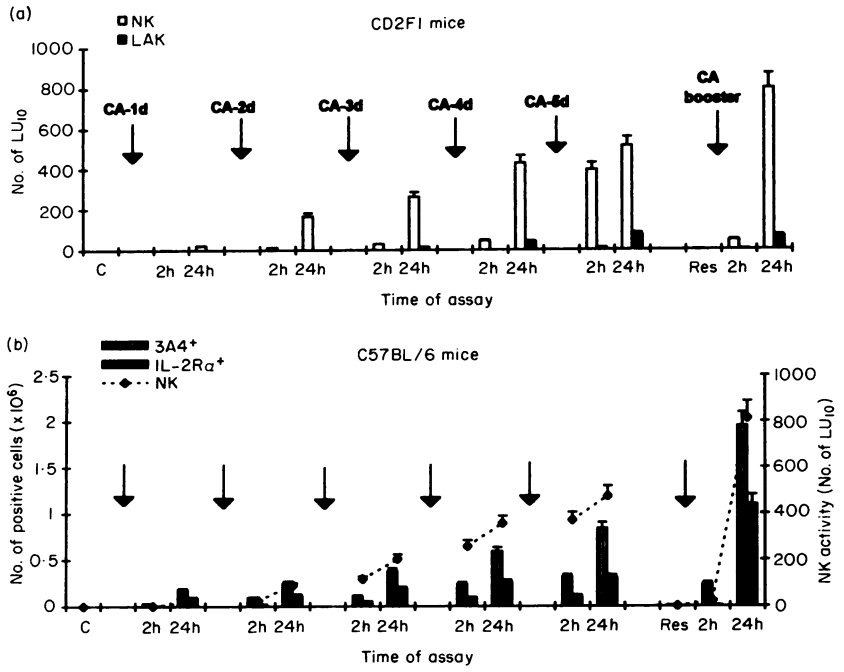

Figure 7. NK/LAK activity of PEC and phenotype of cytotoxic cells at different phases of immune response to CA. (a) PEC were recovered from untreated CD2F1 mice (C), at 2 and 24 hr after every CA stimulation, 30 days after CA-5d (Res) and 2 and 24 hr after CA booster. Lysis of target cells was measured by a 4 hr ^{51}Cr release assay and evaluated as number of LU_{10} per peritoneal exudate. Data are presented as the mean values ± SD of six experiments. (b) PEC were recovered from untreated C57BL/6 mice (C), at 2 and 24 hr after every CA stimulation, 30 days after CA-5d (Res.) and 2 and 24 hr after CA booster. Nylon-wool non-adherent cells were used in the assays. Number of positive cells at cytofluorimetric analysis. Data are presented as the mean values ± SD of four experiments. Lysis of target cells was measured by a 4 hr ^{51}Cr release assay and evaluated as number of LU_{10} per peritoneal exudate. Data are presented as the mean values ± SD of six experiments.

the peak at 24 hr when NK activity was higher in both the primary and anamnestic response.

DISCUSSION

In experimental infection it has been reported that resistance to *C. albicans* is associated with IL-12 driven Th1 subset development.[3,14] Less attention, has however, been given to the nature of the specific immune response to inactivated *C. albicans*. In fact, while its effects on natural immunity cells are well known,[15,16,18] only recently its ability to induce the development of a Th1 response in mice has been suggested.[19]

Investigations to determine the immunological events which influence the Th1 subset development and whether CA is able to generate a specific long-lasting memory, could provide important information for an experimental approach to vaccination.

To this end, we analysed the cytokine pattern expressed in the exudate cells recruited in the peritoneal cavity by CA at different phases of the immune response. In our system, CA priming was followed by an early non-specific phase of immunity which would signal to the specific T-cell response how to develop. In fact, the main events favouring the development of a selected Th subset seem to be the dramatic increase of IL-1β mRNA expression, mainly produced by activated macrophages,[32] with the possible contribution of other cells such as NK[33] and lymphocytes,[34] and the rapid, high and persistent expression of IL-2 mRNA probably produced by naive T cells.[35] IL-2 in the presence of IL-1β, which acts as a co-stimulator,[36] would push naive Th cells to the proliferative phase providing optimum conditions for their expansion and differentiation. Most important is that the synergism between IL-2 and IL-1β would also cause the rapid activation of NK cells, as reported *in vitro*.[37] This seems to occur also in our system as suggested by the fact that after the second CA stimulation, there was a further increase of NK activity concomitant with a rapid induction of IFN-γ mRNA. The expression of IL-2 and IFN-γ, in the absence of IL-4 and IL-5 mRNAs, indicates that the cytokine environment favours the development of the Th1 subset.

The progressive increase of IFN-γ and IL-2 mRNAs with the expression of IFN-γ which persisted up to 24 hr after CA-5d and at this time, the differentiation of specific T lymphocytes, confirmed by the ability of PEC to specifically proliferate *in vitro* in response to CA, seem to indicate that a Th1 response to CA was induced.

However, in our system, Th1 development occurred in the absence of IL-12, a cytokine known to play a key role in directing differentiation of naive Th cells towards the Th1 subset by directly stimulating NK cells to a higher IFN-γ production.[38,39] Consequently, the mechanism that operates in inducing the IFN-γ necessary to drive Th1 development, seems to be the persistent stimulation of NK cells by IL-2 and IL-1β,[40,41] as indirectly confirmed by the progressive increase of NK activity concomitantly with IFN-γ and IL-2 mRNAs levels, following every CA stimulation.

We suggest that during the early phase of the immune response to CA, NK cells are the primary source of IFN-γ because it takes several days for specific lymphocytes to develop, whereas after CA-5d the persistance of IFN-γ mRNA expression up to 24 hr might be due to the fact that at this phase, developed Th1 cells also contribute to IFN-γ production. The high level of IFN-γ probably maintains the IL-10 expression low and transient. In fact, these cytokines have a mutually exclusive regulatory role in the development of the immune response, by reciprocal inhibition depending on their relative concentrations.[42,43] However, even if low and transient, the expression of IL-10 after every CA stimulation, could contrast the development of a Th1 response since this cytokine can inhibit the macrophage antigen presenting cells (APC) function for Th1 cell activation.[44,45] However, recent studies have demonstrated that besides macrophages and Th2 cells, IL-10 is secreted by a distinct subset of peritoneal Ly-5$^+$ B lymphocytes which can also present antigen to Th1 cell clones.[46] Interestingly, B-cell derived IL-10 does not inhibit their APC function for Th1 cells.[46] Therefore, in our system, IL-10 could be produced mainly by Ly-5$^+$ B cells which have been detected in the peritoneal cavity in all phases of the immune response to CA.

In the perspective of a possible use of CA in vaccination, it is very important to know whether the Th1 subset, developed in the primary immune response, conditions the Th subset elicited in the anamnestic response.

In response to CA booster, a cytokine pattern similar to that observed after the development of the primary response was induced.

The selective expression of mRNAs for IL-2 and IFN-γ, newly induced by CA in such a brief time as 2 hr, and the *in vitro* specific proliferative response of PEC to CA would indicate that memory Th1 cells were specifically activated. Concomitant with this afferent phase of antigen recognition by Th1 memory cells, a potent NK/LAK activation, due to the rapid and high induction of IFN-γ and IL-2 by T memory also occurred.

This rapid and efficient recruitment of T lymphocytes and asialo GM1$^+$ cells suggests an influx of T memory cells and NK cells homing on the lymphoid tissues associated with the peritoneal cavity.

This is in agreement with the observations of other authors who suggest that memory T cells, committed to a particular pattern of cytokine secretion during primary immune response, after restimulation produce a similar cytokine profile.[47,48] However, it is probable that the features of the anamnestic response were affected by the experimental models used since other studies have reported that specifically sensitized T cells, after differentiation into memory cells, express a broad repertoire of lymphokines not restricted to the specific subset of the primary response.[49]

In conclusion, since the Th1 response is highly protective against experimental *C. albicans* infection,[3,14] the ability of CA to induce a selected Th1 response, able to persist as a long-lasting immunological memory, provides a rationale for the possible use of this inactivated microorganism, or its immunological active cell wall components, in an experimental approach to vaccination.

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