Cellular Defenses against Toxoplasma gondii in Newborns

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Abstract. Mononuclear phagocytes, particularly macrophages (Mϕ) that have been activated by lymphokines, are the principal defense against intracellular pathogens such as *Toxoplasma gondii*. To determine reasons for the newborns' susceptibility to Toxoplasma infection, we compared: (*a*) the interaction of Toxoplasma with newborns' mononuclear phagocytes (blood monocytes and two types of newborn Mϕ, those derived from blood monocytes or from placental tissue) with adults' blood monocytes and monocyte-derived Mϕ and (*b*) the production of Mϕ-activating lymphokines (MAF) by Concanavalin A (ConA)-stimulated newborn and adult blood mononuclear cells (MC). Newborn and adult monocytes killed Toxoplasma with equal efficiency. Similarly, survival and replication of Toxoplasma were comparable in control newborn and adult Mϕ. Exposure to adult ConA supernatants significantly decreased the survival and replication of Toxoplasma both in adult and newborn Mϕ. In contrast, exposure to cord blood ConA supernatants failed to affect the survival or the replication of Toxoplasma in newborn Mϕ and decreased the replication but not the survival of Toxoplasma in adult Mϕ. Exposure to ConA supernatants of peripheral blood MC from 2-5-d old newborns failed to affect survival or replication of Toxoplasma in newborn or adult Mϕ. Thus, both generation of MAF by newborn blood MC and response to newborn MAF by newborn Mϕ were impaired. Generation of MAF by adult blood mononuclear cells

was not inhibited by cord blood MC nor was generation of MAF by cord blood MC increased by depletion of OKT8 antibody-binding cells, by depletion of adherent cells with or without addition of adult adherent cells, or by addition of indomethacin. Depletion of OKT4 antibody-binding cells abrogated the generation of MAF both by adult and cord blood MC. The activity of adult ConA supernatants was abrogated by dialysis at pH 2 or by addition of anti-γ-interferon but not anti-α-interferon antibody. However, the correlation between antiviral interferon activity and anti-Toxoplasma activity was weak ($r = 0.40$). Enhanced Mϕ anti-Toxoplasma activity was not associated with detectably enhanced superoxide anion generation, nitroblue tetrazolium reduction, or phagolysosome fusion, and was not inhibited by catalase, superoxide dismutase, or mannitol. These results indicate that generation of and response to MAF is decreased in cells from human newborns and that γ-interferon may be the major MAF under these conditions.

Introduction

The human fetus and newborn are unusually susceptible to severe infection with a variety of microorganisms. One such pathogen is *Toxoplasma gondii*, which is an obligate intracellular parasite. Intrauterine Toxoplasma infection occurs as a result of transplacental transmission of parasites from mother to fetus and occurs most frequently in the third trimester (1, 2). The placenta may act as a barrier to transmission of infection in those cases in which active maternal infection is not associated with fetal infection (1, 2).

Mononuclear phagocytes, particularly activated macrophages (Mϕ)[1], are the principal defense against Toxoplasma infection

(1, 3, 4). Mϕ antimicrobial function and Mϕ activation are less efficient in newborn rodents than in adults, and this is associated with increased susceptibility to severe Toxoplasma infection (1). Little information exists regarding host defense mechanisms against intracellular pathogens in human newborns. This report describes in vitro studies of cellular defenses against Toxoplasma in the human newborn and placenta (which is a fetal organ). We observed decreased generation of and response to Mϕ-activating lymphokines (MAF) with newborns' cells compared with adults' cells.

Methods

Reagents

Reagents were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Hanks' balanced salt solution (HBSS), RPMI 1640, calf serum, L-glutamine, penicillin, and streptomycin were obtained from Grand Island Biological Co., Grand Island, NY. When analyzed by limulus amebocyte lysate assay (Pyrotell Associates of Cape Cod, Inc., Woods Hole, MA), the RPMI 1640 contained <0.07–0.10 ng/ml of reference E. coli endotoxin. Hepes buffer was obtained from Calbiochem-Behring Corp., La Jolla, CA. Fetal calf serum (FCS) was obtained from Sterile Systems, Inc., Logan, UT; FCS contained 0.07–0.13 ng/ml endotoxin and lacked detectable (<1:4) Toxoplasma antibody in the Sabin Feldman dye test. Ficoll Hypaque (FH) and Percoll were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. [^{14}C]leucine (348 mCi/mmol), [^{3}H]uracil (40 mCi/mmol), Omnifluor, and Aquasol 2 were obtained from New England Nuclear, Boston, MA. Phytohemagglutinin-P (PHA) was obtained fromm Difco Laboratories, Inc., Detroit, MI. Glutaraldehyde was obtained from Ted Pella, Inc., Irvine, CA. DiffQuik was obtained from Dade Diagnostics, Aguado, PR. Thorium oxide (Thoria-Sol) was obtained from Polysciences, Inc., Warrington, PA. OKT4 and OKT8 monoclonal antibodies were obtained from Ortho Diagnostics, Raritan, NJ. Rabbit complement was obtained from Pel-Freez Biologicals, Rogers, AR. Sheep antiserum to human α-interferon (IF) (G026-502-568) and its control serum were obtained from the National Institutes of Health, Bethesda, MD. Rabbit antiserum to human γ-IF was a generous gift from Dr. M. Langford, University of Texas, Galveston, TX (5). Human α-IF standard (69-19) was obtained from the National Institutes of Health, Bethesda, MD. All glassware was treated at 180°C for 3 h to inactivate endotoxin.

Cell preparations

Blood mononuclear cells. Blood was obtained from healthy adult donors that lacked detectable Toxoplasma antibodies in the Sabin Feldman dye test or indirect fluorescent antibody test. Blood was obtained from the umbilical cord immediately following the delivery of healthy term newborns. In certain experiments, peripheral venous blood was obtained from healthy 2–5-d old newborns. These studies were approved by the Institutional Review Board for Human Subjects of the Children's Orthopedic Hospital and the University of Washington. Blood was anticoagulated with 0.1% potassium EDTA. Mononuclear cells (MC) were obtained by centrifugation on FH as previously described (6), washed twice in HBSS without Ca^{++} and Mg^{++}, and resuspended in RPMI-1640 medium containing 2 mM 1-glutamine, 10 mM Hepes buffer, 100 U/ml penicillin G, 100 μg/ml streptomycin (RPMI), and 20–25% autologous serum or human type AB serum (RPMI + AS) that lacked detectable Toxoplasma antibodies. Cells were enumerated in a hemacytometer and the percentage monocytes was determined by phagocytosis

of neutral red particles (7) and by staining for nonspecific esterase (8). Cell viability was determined by trypan blue dye exclusion.

Monocyte and monocyte-derived Mϕ monolayers. Blood MC were adjusted to a concentration of 1 × 10^6 monocytes per ml in RPMI + AS. Monolayers were established in 16-mm wells of Linbro trays (Linbro Chemical Co., Hamden, CT) as previously described (9); in experiments where monolayers were examined microscopically, these wells contained 15-mm diameter glass coverslips. Monocyte monolayers were infected within 1 h after cells were added to wells. Mϕ were derived by culturing monocytes in vitro for 7–8 d (9). Such monolayers were routinely >95% Mϕ as determined by staining for nonspecific esterase. Monolayer cell numbers were determined as previously described (10, 11) by examining stained coverslips using a calibrated ocular grid.

Placental mononuclear phagocytes. Human placentas from pregnancies of 34–42-wk duration were obtained following uncomplicated vaginal and Caesarian section deliveries. After blood was aspirated from the umbilical vein, the tissue was minced, washed, finely minced, and digested in RPMI 1640 containing 150 units/ml collagenase, 60 units/ml DNAase, and 2% calf serum as previously described (11). Cell preparations were enriched for Mϕ by sequential density gradient separation over FH and Percoll, washed twice in HBSS, resuspended in RPMI + 10% FCS (RPMI + FCS), and enumerated in a hemacytometer. Approximately 0.5–0.75 × 10^6 Mϕ were added to 16-mm Linbro wells, allowed to adhere for 2 h, washed six times with HBSS, and freed of contaminating trophoblastic cells by exposure to 0.1% trypsin for 5–7 min followed by washing six times with HBSS as previously described (11). Such monolayers routinely contain >90–95% Mϕ; >80% are mature Mϕ as indicated by the absence of detectable peroxidase by ultrastructural cytochemistry. In certain experiments, placental Mϕ were cultured in RPMI + AS with comparable results.

Fibroblast monolayers and WISH cells

Human fetal fibroblasts and WISH cells (obtained from Dr. J. Overall, University of Utah) were maintained and cultured as previously described (10).

Organisms and zymosan

Tachyzoites of the RH strain of T. gondii were maintained and processed as previously described (9).

Zymosan was washed three times in phosphate-buffered saline (PBS, pH 7.4) before use, opsonized by incubation for 30 min at 37°C in human serum, washed three times in PBS, and resuspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose.

Lymphokines

Adult and cord blood MC were resuspended in RPMI + AS at a concentration of 5.0 × 10^6 cells/ml. 2.5 ml were added to 15-ml conical tubes (Falcon Plastics, Oxnard, CA). 250 μl of Concanavalin A (ConA) or PHA in the indicated concentrations or medium alone (control) were added, and tubes were incubated at 37°C for the indicated intervals. At that time, an equivalent concentration of ConA or PHA was added to control tubes and all tubes were then centrifuged at 250 g for 10 min. Supernatants were aspirated and those containing ConA were added to 400 mg of Sephadex G-10 for 1 h to adsorb the ConA (12). The Sephadex was then removed by centrifugation at 250 g for 10 min, supernatants were aspirated, sterilized by filtration (0.2 μm Millex filters, Millipore Corp., Boston, MA), and stored at 4°C. Activity in our assays was retained for up to 1 mo under these conditions. Lymphocyte culture supernatants were added to monolayers at the times and concentrations indicated in Results. Lymphocyte transformation for each cell preparation in response to the same stimuli was assessed as previously described (6).

In certain experiments, blood MC were depleted of adherent cells by adherence to plastic Petri dishes (Corning Glass Co., Corning, NY) as previously described (6). Nonadherent cells were then added to nylon wool columns and incubated for 1 h; nonadherent cells were collected as described (13), washed once in HBSS, and resuspended in RPMI + AS. In other experiments, MC were suspended at a concentration of 1×10^7/ml in RPMI + FCS and incubated for 45 min with OKT4 or OKT8 antibody (1:200 final dilution). Then, rabbit serum which had been stored at $-70°C$ was added (1:6 final dilution) as a source of complement, and the cells were incubated for 45 min at 37°C. The cell suspensions were then centrifuged over FH to remove nonviable cells, washed twice in HBSS, and resuspended in RPMI + AS. Such preparations contained <2% OKT4 or OKT8 antibody-containing cells, respectively, as determined by indirect immunofluorescent staining with fluoresceinated (Fab'2) goat antimouse immunoglobulin. Adherent cell-depleted cell preparations, and OKT4 and OKT8-depleted cell preparations were then resuspended at a concentration of 5×10^6 viable cells/ml and stimulated with ConA to generate lymphocyte culture supernatants as described above.

In certain experiments, lymphocyte culture supernatants were dialyzed against pH 2 glycine buffer or PBS for 24 h, then dialyzed for 12 h against PBS, and 12 h against RPMI before use. In other experiments, sufficient antihuman α- or γ-IF antibody to neutralize 3500 units of activity/ml was added to supernatants and incubated for 30 min at 37°C before use.

Mϕ interaction with Toxoplasma

Monolayers were cultured as indicated in Results, washed six times with HBSS, and then 1×10^6 Toxoplasma in 0.5 ml RPMI + FCS were added to each. After 1 h at 37°C in 5% CO_2:95% air, monolayers were washed eight times with HBSS to remove extracellular organisms. Immediately or after reincubation for 20 h in fresh medium, monolayers were fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.0), stained with Diff Quik, and examined microscopically in a blinded manner as previously described (9, 10).

Intracellular survival and replication of organisms was also assessed by monolayer incorporation of [^{3}H]uracil by using a modification of previously described methods (14, 15). Wells without coverslips were infected for 1 h as described above, washed, and 1 ml of fresh medium and 10 μCi of [^{3}H]uracil were added to each. After incubation for 18 h, monolayers were washed eight times with HBSS and solubilized in 0.1% Triton X-100. An aliquot was saved for determination of protein content by a modification of the Lowry method (16) using bovine serum albumin as the standard; 10% ice-cold TCA was added to the remainder and the precipitate was collected on filter papers, dried, added to vials containing Omnifluor, and the associated radioactivity determined in a liquid scintillation spectrometer.

Effects of inhibitors

The oxygen metabolite scavengers superoxide (O_2^-) dismutase (SOD, 2.5 mg/ml, 2,800 U/mg protein), catalase (0.25 mg/ml, 34,000 U/mg protein), and mannitol (50 mM) were added to monolayers for 4 h before and during the assays where indicated.

O_2^- generation and nitroblue tetrazolium (NBT) reduction

O_2^- release by Mϕ monolayers was assessed in response to optimal concentrations of phorbol myristate acetate (PMA, 1.6 μg/ml) or opsonized zymosan (2.5 mg/ml) by SOD-inhibitable reduction of ferricytochrome C as previously described (17), except that monolayers were in 16-mm rather than 35-mm wells. NBT reduction by Mϕ-phagocytosing Toxoplasma was assessed microscopically as previously described (9).

Phagolysosome fusion

Mϕ were cultured in 60-mm Petri dishes (Corning Glass Works) and lysosomes were labeled with thorium oxide 24 h before the assay as described by Goren et al. (18). Mϕ monolayers were then washed and infected with Toxoplasma for 1 h as described above. One set of dishes was immediately fixed in 2.5% glutaraldehyde in Na-cacodylate buffer; another set was incubated for an additional 1-2 h and then fixed. Dishes were washed, processed, and examined by transmission electron microscopy as previously described (11). Results with monolayers that were processed 1 or 2 h after infection were comparable and they were pooled.

Interferon assays

The IF content of lymphocyte culture supernatants was assayed by a previously described plaque reduction assay using vesicular stomatitis virus and human foreskin fibroblasts (19). A standard preparation of human α-IF was used in each assay for comparison.

Statistics

The significance of the differences between means was evaluated by two-tailed Student's t test or by paired t test as appropriate. The significance of differences between nonparametric data was examined by Fisher's exact test or by the chi-square test for small and large samples, respectively (20).

Results

Interaction of Toxoplasma with blood monocytes. During the 1-h infection period, comparable numbers of freshly isolated blood monocytes from adults and newborns phagocytosed Toxoplasma (Fig. 1); the mean numbers of intracellular Toxoplasma per infected cell were also comparable (not shown). Toxoplasma were cleared at a similar rate by both. Examination of cytocentrifuge-prepared slides of the supernatants (15) indicated that the decrease in the percentage of infected cells was not due to a selective loss of infected cells from the monolayers. Toxoplasma replicated poorly in both types of monocytes (Fig.
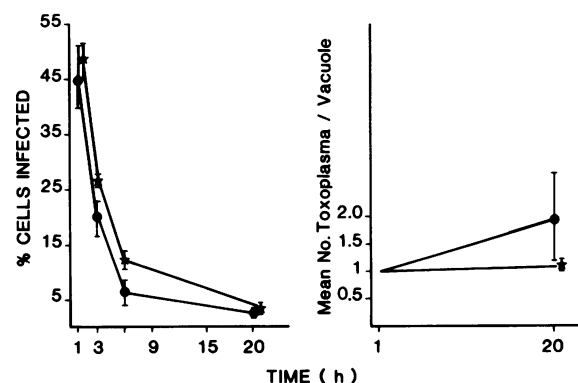
Figure 1. Interaction of Toxoplasma with freshly isolated monocytes. Adult (●) and cord blood (*) monocytes were infected with Toxoplasma tachyzoites, and at 1 h, extracellular organisms were removed and the infected monolayers were incubated for the indicated intervals. The percentage of infected monocytes and mean no. of Toxoplasma per vacuole were determined by light microscopy of Diff Quik stained smears. Results are the mean±SE of four experiments.

1); the mean number of parasites per vacuole at 20 h in adults' and newborns' monocytes was not significantly different.

Interaction of Toxoplasma with freshly isolated placental macrophages. When placental macrophages were infected immediately or within 16 h of isolation, survival and replication of Toxoplasma were greater than in freshly isolated blood monocytes but comparable with that in Mϕ derived from adult blood monocytes that had been cultured in vitro for 4–5 d (Fig. 2). Exposure of adult monocyte-derived Mϕ to collagenase and trypsin under conditions used to isolate placental Mϕ did not affect uptake, survival, or replication of Toxoplasma in these cells (not shown). If placental Mϕ and adult monocyte-derived Mϕ were cultured in vitro for a longer period of time (7–9 d), phagocytosis ($P < 0.001$ and <0.02, respectively) and intracellular replication ($P < 0.001$) of Toxoplasma increased significantly in both (Fig. 2).

Conditions for enhancement of Mϕ anti-Toxoplasma activity by blood MC culture supernatants. Supernatants from mitogen or antigen-stimulated adult lymphocyte cultures have been reported to enhance the anti-Toxoplasma activity of human monocyte-derived Mϕ (21, 22). Under our conditions, ConA-stimulated adult blood MC culture supernatants (ConA sups) did not consistently enhance the anti-Toxoplasma activity of adult Mϕ derived from monocytes that had been cultured in vitro for <96 h before they were exposed to supernatants for
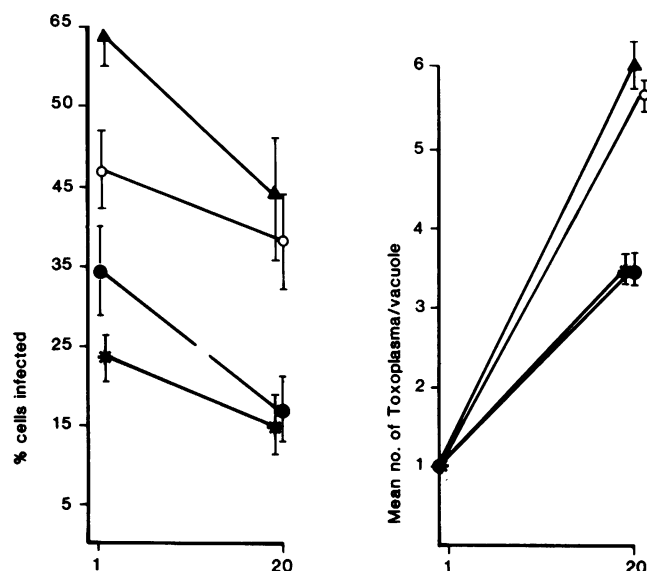

Figure 2. Interaction of Toxoplasma with adult Mϕ derived from the culture of blood monocytes in vitro for 4–5 d (●) or 7–9 d (o) and freshly isolated placental Mϕ (*) or placental Mϕ cultured in vitro for 7–9 d (▲). Mϕ were infected and assessed as described for Fig. 1. Results are the mean±SE of 8 (adult Mϕ) or 10 (placental Mϕ) experiments. After the 1-h infection period, the mean number of Toxoplasma per infected Mϕ was 1.6±0.1, 1.8±0.1, 1.2±0.1, and 2.1±0.4 for 4–5-d and 7–9-d adult Mϕ and freshly isolated and 7–9-d placental Mϕ, respectively.

48–72 h; unexposed control Mϕ and Mϕ exposed to the adult ConA sups both cleared ~50% of Toxoplasma between 1 and 20 h, and the mean number of Toxoplasma per vacuole was 3.5±0.2 and 3.4±0.4, respectively. However, adult monocytes that had been cultured in vitro for >96 h before addition of supernatants, developed enhanced anti-Toxoplasma activity when exposed to adult ConA sups (Fig. 3). Enhancement of placental Mϕ anti-Toxoplasma activity also occurred only when cells were cultured in vitro for >72 h before exposure to adult ConA sups. However, this period before placental Mϕ would respond to adult ConA sups may be due in part to the effects of the isolation procedure. Mϕ derived from adult monocytes that had been cultured in vitro for 5 d and then exposed to collagenase and trypsin under conditions used to isolate placental Mϕ lost their ability to respond to adult ConA sups with increased anti-Toxoplasma activity for at least 72 h. When adult monocyte-derived Mϕ were exposed to adult ConA sups before treatment with collagenase and trypsin, and then infected, enhanced anti-Toxoplasma activity was observed (not shown). This suggests that enzyme treatment interferes with the action of adult ConA sups on Mϕ rather than impairing Mϕ anti-Toxoplasma activity directly.

In preliminary experiments, exposure of adult or placental Mϕ to adult ConA sups at a final concentration of 30% (compared with 10 or 50%) for 3 d (compared with 2 or 4 d) resulted in the greatest enhancement of Toxoplasma clearance and the greatest inhibition of intracellular replication. Daily addition of 10–30% adult ConA sups for 3 d had an effect similar to a single addition of 30% supernatants for 3 d before the assay. Also, the use of 40 μg/ml (compared with 20 or 80 μg/ml) of ConA during production of adult ConA sups resulted in supernatants that optimally enhanced Mϕ anti-Toxoplasma activity (not shown). These conditions were used for the subsequent studies unless otherwise indicated.

Effects of ConA supernatants from adults and newborns on survival and replication of Toxoplasma. Replication of Toxoplasma in adult or cord blood Mϕ or placental Mϕ was significantly inhibited after exposure of these cells to adult ConA sups (Fig. 3 *A*) or to supernatants from Toxoplasma antigen-stimulated blood MC from immune adults (not shown). In contrast, only in adult Mϕ was replication of Toxoplasma significantly inhibited after exposure to cord blood ConA sups. Further, replication of Toxoplasma was significantly less in adult Mϕ and placental Mϕ that were exposed to adult ConA sups compared with cord blood ConA sups (Fig. 3 *A*). Replication of Toxoplasma in adult Mϕ, cord blood Mϕ, or placental Mϕ was not affected by exposure to unstimulated adult or cord blood MC supernatants (mean no. of Toxoplasma per vacuole, 4.9±0.4, 5.2±0.6, and 6.4±0.6, respectively).

Exposure to adult or cord blood ConA sups did not affect the percentage of adult, cord blood, or placental Mϕ that were infected or the number of Toxoplasma per infected cell following the 1 h challenge (Fig. 3 *B*). Significantly fewer adult, cord blood, or placental Mϕ that were exposed to adult ConA sups remained infected by 20 h compared with each type of untreated
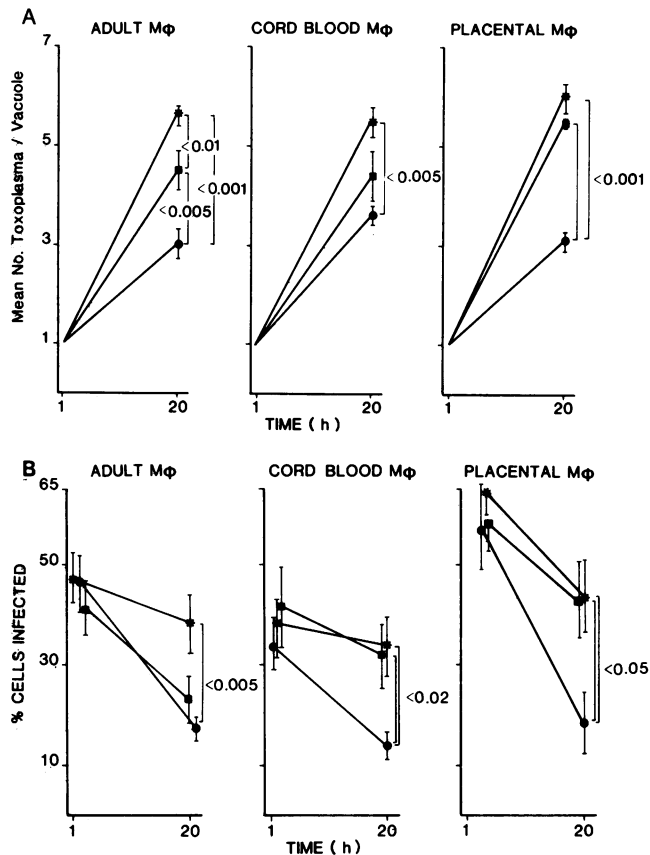
Figure 3. Effects of ConA-stimulated adult and cord blood mononuclear cell supernatants on replication (A) and survival (B) of Toxoplasma in adult Mϕ, cord blood Mϕ, and placental Mϕ. Mϕ were exposed to adult ConA sups (●), cord blood ConA sups (■), or medium alone (∗). Mϕ were infected and assessed as described for Fig. 1. Results with Mϕ that were exposed to unstimulated adult or cord blood sups were comparable with those with medium alone. Results are the mean±SE of 23-24, 4-6, and 6-8 experiments, respectively. After the 1-h infection period, the mean no. of Toxoplasma per infected cell for Mϕ exposed to medium alone, adult ConA sups, or cord blood ConA sups was 1.8±0.1, 1.7±0.1, and 1.6±0.1 for adult Mϕ; 2.1±0.2, 2.0±0.2, and 2.1±0.3 for cord blood Mϕ; 2.1±0.4, 1.6±0.4, and 2.0±0.2 for placental Mϕ, respectively.

Mϕ or to cord blood or placental Mϕ that were exposed to cord blood ConA sups (Fig. 3 B). In contrast, with each type of Mϕ, there was no significant difference in the percentage of infected Mϕ at 20 h between controls and cells exposed to cord blood ConA sups.

It is unlikely that these differences were due to increased or selective cell loss from monolayers that were exposed to adult ConA sups. The number of each type of Mϕ in untreated monolayers and monolayers exposed to adult or cord blood ConA sups was comparable after 1 h (1.46±0.13, 1.35±0.13, and 1.52±0.15 × 10^5, respectively) and 20 h (1.03±0.26, 1.08±0.30,

0.94±0.18 × 10^5, respectively). Similarly, when the detached cells in 20-h culture supernatants from three to four representative experiments with adult Mϕ were examined in cytocentrifuge-prepared slides, the percentage of infected cells and the mean no. of Toxoplasma per vacuole paralleled those of the respective monolayers; the mean percentage of infected cells was 33, 12, and 8, and the mean no. of Toxoplasma per vacuole was 4.0, 1.6, and 2.1 in untreated and adult or cord blood ConA sups exposed Mϕ, respectively.

Similar results were obtained when survival and replication of Toxoplasma were assessed by uptake of [^{3}H]uracil by infected Mϕ. Uptake of [^{3}H]uracil by Toxoplasma-infected adult Mϕ that had been exposed to adult control sups (2215±319 cpm/μg protein) and to cord blood ConA sups (1999±1046 cpm/μg protein) were comparable, whereas uptake of [^{3}H]uracil by infected Mϕ that were exposed to adult ConA sups (1240±364 cpm/μg protein) was significantly ($P < 0.025$) less. Monolayer protein contents were comparable. Similarly, uptake of [^{3}H]uracil by placental Mϕ that had been exposed to adult but not to cord blood ConA sups was significantly ($P < 0.05$) less than that by Mϕ exposed to adult control sups (not shown). [^{3}H]uracil uptake by uninfected Mϕ or Toxoplasma alone was <1% of that by control-infected Mϕ.

To determine if peripheral blood MC from newborns, like cord blood MC, were deficient in production of factors that enhance Mϕ activity against Toxoplasma, peripheral blood MC were obtained from 2-5-d old newborns and used to produce ConA sups. In three experiments each, exposure to newborn peripheral blood MC ConA sups did not decrease the survival or replication of Toxoplasma at 20 h in adult Mϕ (39.4±2.7% infected Mϕ, 5.7±0.1 Toxoplasma/vacuole) or in placental Mϕ (46.0±5.2% infected Mϕ, 5.8±0.1 Toxoplasma/vacuole) compared with control Mϕ (31 and 43% infected Mϕ and 5.5 and 4.9 Toxoplasma/vacuole, respectively).

Basis for differences between the effects of adult and cord blood ConA sups on Mϕ activation against Toxoplasma. Decreased activation of Mϕ by cord blood ConA sups was not associated with a failure of lymphocytes to transform in response to ConA, since uptake of [^{3}H]thymidine by ConA-stimulated cord blood MC (50107±5864 cpm) was comparable to that by ConA-stimulated adult blood MC (54971±6058 cpm). Uptake of [^{3}H]thymidine by unstimulated cord blood MC (4208±764 cpm) was greater than that by adult blood MC (1079±200 cpm). Varying the concentration of cord blood ConA sups added to adult Mϕ culture medium did not affect the results; the mean no. of Toxoplasma per vacuole was 5.6, 5.3, 5.0, and 5.4 with addition of 5, 10, 30, and 50% cord blood ConA sups, respectively ($n = 2-5$). Similarly, use of 20 or 80 μg/ml of ConA, instead of 40 μg/ml of ConA, to stimulate cord blood MC did not affect results (not shown). Furthermore, supernatants of cord blood MC that were stimulated with PHA were not more effective at inducing toxoplasmastatic activity in adult Mϕ than were ConA-stimulated cells from the same subject (mean no. of Toxoplasma

per vacuole: cord blood PHA sup = 5.4, cord blood ConA sup = 5.1).

To determine if a subpopulation of cord blood MC accounted for this difference, we first determined the subpopulation necessary for production of MAF by adult blood MC. ConA supernatants of adult blood MC that had been treated with OKT4 antibody and complement were significantly less active than supernatants prepared under the standard conditions (conventional supernatants) both in their effects on replication (Table I) and on survival (not shown) of Toxoplasma in adult Mϕ; ConA-stimulated OKT4-depleted blood MC took up ~50% as much [^{3}H]thymidine as did undepleted blood MC (not shown). In contrast, treatment of adult blood MC with OKT8 antibody and complement (Table I) or depletion of adherent cells (1% esterase-positive cells remained, not shown) were without effect. These results suggested that OKT4 antibody-binding cells were the major source of the MAF in adult ConA sups.

Similar results were observed with cord blood ConA sups; treatment of cord blood MC with OKT8 antibody and complement was without effect, whereas ConA supernatants of cells treated with OKT4 antibody and complement were even less effective ($P < 0.05$) than conventional cord blood ConA sups

Table I. Effects of Different Culture Conditions on Generation of Macrophage-Activating ConA Supernatants by Blood Mononuclear Cells

Adult ConA supernatant	n	Δ Mean no. Toxoplasma per vacuole*	P value
OKT8-depleted	3	−0.13±0.32	NS
OKT4-depleted	3	+1.60±0.40	<0.05
Adult and cord blood MC cocultured	3	+0.13±0.23	NS
Cord blood ConA sup added to adult ConA sup	5	+0.14±0.17	NS
Cord blood ConA supernatant			
Adherent cell-depleted	5	−0.42±0.27	NS
Indomethacin (0.5 μg/ml) added	3	−0.58±0.43	NS
OKT8-depleted	4	+0.20±0.11	NS
OKT4-depleted	3	+1.05±0.23	<0.05

Conventional adult and cord blood ConA-stimulated sups were produced as described in Methods. Where indicated, supernatants were produced in a similar manner either by using cell preparations deleted of adherent cells or of OKT8 or OKT4 antibody-binding cells, or by using cocultures of equal numbers of adult and cord blood blood MC. Supernatants were added to adult monocyte-derived Mϕ 72 h before they were infected with Toxoplasma.
* Results are the mean no. of Toxoplasma per vacuole of monolayers exposed to study supernatants minus that for monolayers exposed to the appropriate conventional supernatant. In the experiments with newborn ConA sups, the mean no. of Toxoplasma per vacuole was 5.7±0.2 and 4.4±0.3 in unexposed and conventional cord blood ConA sup exposed Mϕ; in the experiments with adult supernatants, the mean no. of Toxoplasma per vacuole was 5.7±0.3 and 3.4±0.3 in unexposed and conventional adult ConA sup exposed Mϕ.

Table II. Effects of Different Treatments on Macrophage Activation by ConA Supernatants of Adult Blood Mononuclear Cells

Treatment of adult ConA supernatants	n	Δ Mean no. Toxoplasma per vacuole*	P value
Dialyzed at pH 2	4	+1.50±0.37	<0.05
Dialzyed at pH 7.4	2	−0.10±0.10	NS
Treated with anti-γ-IF	3	+1.90±0.35	<0.05
Treated with anti-α-IF	3	+0.30±0.42	NS

ConA supernatants were produced as described in Methods. They were treated as indicated before use. Supernatants were added to adult monocyte derived Mϕ 72 h before they were infected with Toxoplasma.
* Results were calculated as in Table I. The mean no. of Toxoplasma per vacuole was 5.6±0.3 and 3.5±0.2 in unexposed and conventional adult ConA sup exposed Mϕ.

(Table I). Because adherent cells may suppress lymphocyte responses by secretion of prostaglandin E (23), we assessed the effects of adherent cell depletion (1% esterase-positive cells remained) or addition of indomethacin. The activity of such supernatants was not signficantly different than that of conventional cord blood ConA sups (Table I). Addition of 10% adult adherent cells to nonadherent newborn MC was also without effect (not shown). Cord blood MC did not inhibit production of MAF when co-cultured with adult blood MC and ConA (Table I). Similarly, cord blood ConA sups did not appear to contain a soluble inhibitor of MAF (24), since the toxoplasmastatic effect of adult ConA sups was not affected by addition of cord blood ConA sups (Table I).

Results similar to these were obtained in selected experiments using placental Mϕ instead of adult Mϕ and cord blood ConA sups under variable conditions as described above.

Partial characterization of MAF in culture supernatants. Dialysis of adult ConA sups at pH 7.4 did not affect Mϕ anti-Toxoplasma activity (Table II). In contrast, dialysis of adult ConA sups at pH 2 abrogated their ability to activate Mϕ to inhibit Toxoplasma replication. Further, addition of antihuman γ-IF but not antihuman α-IF abrogated the activity of adult ConA sups on replication (Table II) and on survival of Toxoplasma (not shown). Undiluted adult ConA sups contained more IF (1815±1078 units/ml, n = 4) than cord blood ConA sups (760±559 units/ml). However, there was substantial variability in IF content both of adult and of cord blood ConA sups, and the correlation between supernatant IF titer and its effect on inhibition of Toxoplasma replication in Mϕ was weak (r = 0.40). Control supernatants or supernatants of OKT4-depleted cells contained <100 units/ml of IF.

These supernatants did not induce anti-Toxoplasma activity in nonphagocytic cells, since exposure to adult ConA sups did not impede replication of Toxoplasma in human fibroblasts as indicated by uptake of [^{3}H]uracil by infected cells (control cells = 4227±1364 and adult ConA sup exposed cells = 4233±1481 cpm/μg protein, respectively; n = 3). Similar results were obtained with WISH cells (not shown).

Mechanism of enhanced Mφ antimicrobial activity. Previous studies by ourselves (9, 10) and by Murray and co-workers (25–28) have implicated reactive oxygen metabolites as one mediator of Mφ anti-Toxoplasma activity. However, exposure of adult or placental Mφ to adult ConA sups under the same conditions that enhanced anti-Toxoplasma activity did not increase O_2^- release in response to PMA or zymosan or increase NBT reduction around ingested Toxoplasma (Table III). In contrast, freshly isolated monocytes released 2.5 to 5 times as much O_2^- as untreated or ConA sup-exposed Mφ, and reduced NBT around ingested Toxoplasma. Further, addition of oxygen metabolite scavengers before and during the assay did not inhibit replication (2.7±0.2, 2.2±0.3, 2.5±0.3, and 2.5 Toxoplasma per vacuole in monolayers without or with catalase, mannitol, or SOD, respectively) or survival (not shown) of Toxoplasma in supernatant exposed Mφ (n = 3). Similarly, fusion of thorium oxide-labeled lysosomes with Toxoplasma-containing phagosomes was slightly but not significantly greater in Mφ exposed

Table III. Effect of ConA-stimulated Adult Blood MC Supernatants on O_2^- Production and NBT Reduction by Adult and Placental Mφ

	O_2^-* production		NBT reduction‡ Toxoplasma
	PMA	Zymosan	
	nmol/mg/min		*% cells NBT+*
Adult Mφ			
Control	1.14±0.26	0.48±0.13	<4%
Adult ConA sup	1.29±0.39	0.44±0.13	<4%
Placental Mφ			
Control	1.87±0.38	1.11±0.17	<4%
Adult ConA sup	1.79±0.35	1.19±0.31	<4%

* Mφ were cultured and exposed to adult ConA sups as described for Fig. 3. Then, monolayers were washed and overlayed with Krebs-Ringer's phosphate buffer (pH 7.4) containing 5.5 mM glucose, 75 μM ferricytochrome *C*, and PMA (1.6 μg/ml) or opsonized zymosan (4 μg/ml) in optimal concentrations. SOD (50 μg/ml) was added to control wells. After 90 min, supernatants were aspirated and assayed for SOD-inhibitable reduction of ferricytochrome *C*. Results are the mean±SE for six (adult Mφ) and three (placental Mφ) experiments. Control and adult ConA sup-exposed monolayers had comparable protein content. Under these conditions, freshly isolated adult monocytes released 2.60±0.23 and 2.17±0.01 nmol O_2^- mg/min in response to PMA and zymosan, respectively.
‡ Monolayers were infected as described in Fig. 1 in the presence of 0.2 mg NBT for 1 h. Results are the mean±SE percentage of Mφ-containing Toxoplasma stained with formazan for three experiments. Under these conditions 94.0±3.0 and 89.5±4.5% of freshly isolated monocytes that ingested zymosan or Toxoplasma, respectively, reduced NBT around the particle.

to adult ConA sups (16.0±3.7%) than in controls (6.5±2.5%, n = 4; P < 0.1, >0.05).

Discussion

Anti-toxoplasma activity and activation of newborn and adult Mφ. We found that the anti-Toxoplasma activity of newborn monocytes and Mφ was comparable with that of adult cells, and that both adult and newborn Mφ developed enhanced anti-Toxoplasma activity after exposure to adult ConA sups. Our results with adult Mφ and adult ConA sups are comparable with those reported previously (9, 15, 21, 22, 29).

In contrast, cord blood ConA sups were significantly less effective than adult ConA sups at enhancing adult Mφ anti-Toxoplasma activity, and failed to enhance newborn Mφ anti-Toxoplasma activity. Further, supernatants of ConA-stimulated peripheral blood MC from 2–5-d old newborns did not enhance adult or newborn Mφ anti-Toxoplasma activity. Thus, both generation of and response to MAF were impaired in newborn cells.

In contrast to MAF generation, thymidine uptake, an index of cell replication, by ConA-stimulated cord blood MC was comparable with that by adult blood MC. Others have observed decreased generation of lymphokines by newborn blood MC compared with adult blood MC despite comparable thymidine uptake. Newborn blood MC produce less Mφ migration inhibition factor (33, 34), lymphotoxin (35), and lymphocyte-derived chemotactic factor (LDCF, 36) than do adult blood MC. Bryson et al. (37) found that newborn blood MC produced less γ-IF than did adult blood MC, whereas Handzel et al. (38) did not. Production of leukocyte migration inhibition factor by newborn cells is not significantly less than by adult cells (34, 38, 39).

Decreased production of MAF by cord blood MC did not appear to be due to excessive suppression. OKT4 antigen-positive cells were the major producer of MAF and γ-IF both in adult and cord blood MC preparations. Although others (40, 41) have found that newborn T suppressor cells (T-γ or OKT8-positive) inhibit autologous or allogeneic lymphocyte response in vitro, we were unable to demonstrate such an effect on MAF production by cord blood MC. We did not find prostaglandin- or monocyte-mediated suppression of cord blood MC lymphokine production, nor did cord blood MC inhibit production of MAF by adult blood MC. Further, addition of adult adherent cells to cord blood nonadherent cells did not enhance supernatant anti-Toxoplasma activity. These results suggest that MAF-producing lymphocytes may be deficient in number or in MAF- or interleukin 2- (42) producing capacity in cord blood.

Cord blood ConA sups were significantly less effective at inducing anti-Toxoplasma activity in newborn Mφ than in adult Mφ. Similarly, (*a*) newborn natural killer (NK) cell activity against herpes simplex virus-infected targets is less consistently enhanced by α-IF than is adult NK cell activity (43); (*b*) cells from newborns are less susceptible to the induction of antiviral activity by α-IF and γ-IF than are adult cells (44); and (*c*) newborn LDCF is a less effective stimulus for migration of newborn

than adult monocytes, whereas adult LDCF stimulates comparable migration of both (36). These results suggest that cord blood MC are relatively deficient in generation of lymphokines and that newborn cells are less sensitive to the effects of certain of these lymphokines.

We did not find differences between cells obtained following vaginal or Caesarian delivery either: (a) in the effects of cord blood ConA sups on $M\phi$, or (b) in the response to adult or cord blood ConA sups by cord blood or placental $M\phi$. This differs from results with $M\phi$ release of endogenous pyrogen, which is greater (45), and granulocyte oxidative metabolic response (46) and lymphocyte-mediated cytotoxicity (46), both of which are lower, in cord blood from newborns delivered after labor than after Caesarian delivery without labor.

Partial characterization of MAF. Our results suggest that part or all of the MAF activity in our ConA supernatants was γ-IF or that MAF was dependent on γ-IF to activate $M\phi$ under our conditions. The activity was lost by dialysis overnight at pH 2 but not at pH 7.4 and by preincubation with anti-γ-IF but not anti-α-IF (47). Control supernatants or supernatants of OKT4-depleted ConA-stimulated adult blood MC lacked macrophage-activating activity and had <100 units/ml of IF. Previous studies have yielded conflicting results regarding the nature of MAF (21, 30, 48, 49). However, an increasing body of data suggests that γ-IF may account for most if not all the MAF activity of similar lymphokine preparations in assays of $M\phi$ activity against Toxoplasma (50, 51), other microbes (52–54), and tumor cells (55–57). Further, consistent with the report by Nathan et al. (51), we have found that exposure of adult $M\phi$ and placental $M\phi$ to recombinant γ-IF decreases survival and replication of Toxoplasma in these cells (unpublished observations). These data are consistent with a major role for γ-IF in mediating enhanced $M\phi$ anti-Toxoplasma under our conditions.

Certain data suggest that factors other than γ-IF may play a role in $M\phi$ activation under our conditions. The anti-Toxoplasma activity of fibroblasts and WISH cells was not affected by exposure to adult ConA sups under our conditions or those used by Jones and Byrne (30). However, it is possible that these cells lack the appropriate mechanisms for transducing anti-Toxoplasma but not antiviral activity. Using different conditions, other workers have found that Toxoplasma replication in human fibroblasts (58) or mouse L cells (28) was impaired by γ-IF or by lymphokines, respectively. The anti-Toxoplasma activity in our studies and those of Nathan et al. (51) did not correlate strongly with antiviral activity. In addition, Nathan et al. (51) found that recombinant γ-IF had more potent $M\phi$-activating activity per unit of antiviral activity than did partially purified γ-IF or γ-IF-containing lymphocyte culture supernatants. They proposed that this discrepancy might be related to: (a) the presence of inhibitory or costimulatory factors in lymphocyte supernatants and in certain preparations of partially purified γ-IF; and (b) less potent MAF activity per unit of antiviral activity in natural γ-IF, which is glycosylated, than in recombinant γ-IF, which is not glycosylated. The second possibility was fa-

vored by their data (51). Similarly, one might speculate that the more profound deficiency in activity of cord blood ConA sups with newborn $M\phi$ compared with adult $M\phi$ might be due to: (a) the presence of factors which specifically inhibit, or the absence of factors which specifically enhance newborn compared with adult $M\phi$ anti-Toxoplasma activity; (b) differences in glycosylation of γ-IF in cord blood compared with adult blood ConA sups that decrease effectiveness with newborn $M\phi$ relative to adult $M\phi$.

Although low levels of endotoxin (<0.07–0.10 ng/ml) were present in some of our culture medium, these concentrations were much lower than 100 μg/ml of endotoxin, which Jones and Byrne (30) found to be a necessary cofactor for enhancement of resident mouse peritoneal $M\phi$ activity against Toxoplasma by lymphocyte supernatants. Further, our results were comparable with cells cultured in medium lacking detectable endotoxin, in medium containing $\sim$0.1 ng/ml of endotoxin, and in medium to which polymyxin B (5 μg/ml; unpublished observations) was added to inhibit the effects of endotoxin (31). Thus, although very low (1–100 pg/ml) concentrations of endotoxin may effect the ability of human $M\phi$ to generate oxygen radicals (32), it is unlikely that endotoxin was a necessary cofactor for the enhancement of $M\phi$ anti-Toxoplasma activity by adult ConA sups.

Mechanisms for the enhanced anti-Toxoplasma activity of activated $M\phi$. The mechanism for the enhanced $M\phi$ anti-Toxoplasma activity was not fully defined by our study. Our results suggest that enhanced generation of reactive oxygen intermediates was not the principal mechanism. O_2^- production by PMA or zymosan stimulated $M\phi$ was not increased in adult ConA sup-exposed cells. Nakagawara et al. (59) and others (28, 50, 60) found that exposure of adult human monocyte-derived $M\phi$ to adult ConA sups did increase release of H_2O_2; however, in the studies of Nakagawara et al. (59), O_2^- generation was only slightly increased under the same conditions. We have also found that exposure to adult ConA sups augments $M\phi$ H_2O_2 release to a greater extent than O_2^- release (unpublished observations). The reason for the difference in detection by these assays is unclear (59). However, Toxoplasma ingestion failed to trigger detectable NBT reduction and oxygen metabolite scavengers did not reverse the anti-Toxoplasma activity of $M\phi$ exposed to adult ConA sups. Thus, it appears unlikely that reactive oxygen metabolites mediated the enhanced $M\phi$ anti-Toxoplasma activity. Further, phagolysosome fusion in adult ConA sup-exposed $M\phi$ was not significantly ($P > 0.05$, <0.1) greater than in controls. We (9, 10) and others (28, 30) have previously concluded that the oxidative burst and lysosomal fusion are not required for $M\phi$ anti-Toxoplasma activity but may facilitate killing or inhibition of Toxoplasma by mononuclear phagocytes. This conclusion is supported by the observation that the anti-Toxoplasma activity of $M\phi$ activated under the conditions described by Murray et al. (28) and Nathan et al. (51), which reduced NBT during ingestion of Toxoplasma and generated increased amounts of H_2O_2 in response to PMA, was somewhat greater than that of $M\phi$ activated under our conditions. It is possible that other

mechanisms such as vacuolar acidification or entry of small cytoplasmic proteins without phagolysosome fusion (28) may in part mediate the anti-Toxoplasma effects of lymphokine-exposed Mφ. Since Toxoplasma, like viruses, is an obligate intracellular pathogen, it is also possible that the decreased survival and replication is due to interference with the as yet imprecisely defined metabolic support, which is normally provided by the cell to the parasite.

It is not possible to determine from our in vitro studies the relative role which monocytes, macrophages, and lymphokine-induced macrophage activation play in control of Toxoplasma infection in vivo. It is possible that these findings may relate to the increased severity of infection with Toxoplasma and other intracellular pathogens in human fetuses and newborns. Although the anti-Toxoplasma activity of newborn blood monocytes was comparable with that of adult blood monocytes, these cells are few in number compared with tissue Mφ and probably play a role only in limiting the initial dissemination of parasites (10, 15). Ultimate control of infection is probably dependent both on development of Mφ activation and specific antibody (3). Decreased generation of MAF by fetal lymphocytes and relatively decreased responsiveness to newborn MAF by placental Mφ may allow unchecked replication of Toxoplasma in this organ. Unchecked replication of Toxoplasma in the placenta would contribute to subsequent spread of infection to the fetus, which occurs in ~65% of pregnancies in which maternal Toxoplasma infection is acquired in the third trimester (1). Similarly, replication in the newborn's tissue macrophages and constitutive cells may be greater than in adults, leading to the greater incidence of significant tissue damage in newborns than in adults. Since disease in newborns is more severe when acquired early in utero it is possible that more severe deficits in macrophages activation or deficits in monocyte function would be found if similar assays were performed using cells from fetuses early in gestation. Interestingly, Toxoplasma infection does not appear to produce more severe infection in the pregnant than in the nonpregnant patient (1, 2), and the MAF activity of ConA-stimulated blood MC from second and third trimester pregnant women was comparable with that from other adults (unpublished observations). Furthermore, decreased production of and response to γ-IF might also contribute to the newborn's greater susceptibility to infection with viruses such as herpes simplex and cytomegalovirus, susceptibility which may be mediated at least in part by Mφ and NK cells and be modulated by IF (43, 61–64). Since newborn Mφ do respond to MAF (γ-IF) from adult cells, enhancement of these antimicrobial functions in newborns by exogenous IF or other immunomodulators may be possible.

Acknowledgments

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