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Sera of Patients with High Titers of Immunoglobulin G against *Toxoplasma gondii* Induce Secretion of Tumor Necrosis Factor Alpha by Human Monocytes

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***Toxoplasma gondii* alone does not induce tumor necrosis factor alpha (TNF-α) secretion by human monocytes and macrophages. Nevertheless, sera from infected patients with high titers of specific immunoglobulin G antibodies against *T. gondii* induce TNF-α secretion, which is significantly higher than the corresponding induction by negative sera ($P < 0.05$). After incubation with the positive serum, parasites also induce secretion of this cytokine, but TNF-α levels are lower (11.4 to 71.8%) than those obtained with positive serum alone. Therefore, this secretion seems to be elicited in part by antibody-*T. gondii* complexes and/or another unidentified factor(s), probably different from lipopolysaccharide, interleukin-1, TNF-α, and gamma interferon. In this study, monocytes secreted more TNF-α into the culture fluid than macrophages did ($P < 0.05$), and no correlation was observed between secretion of this cytokine by the monocytes and the intracellular multiplication of the parasites, evaluated by [^{3}H]uracil incorporation. Sera from patients with other infectious diseases did not induce secretion of TNF-α; however, serum free of antibodies to *T. gondii*, obtained from patients with leishmaniosis, also stimulated secretion of the cytokine.**

Toxoplasma gondii is an obligate intracellular parasite; humans are an intermediate host in its life cycle. It can cause severe disease after congenital infection and in immunocompromised patients. Intracellular multiplication of the parasite leads to cyst formation in immunocompetent individuals (9). In patients with AIDS, toxoplasmosis reactivation is frequent and serious (5), since immunity against *T. gondii* is mainly T-cell mediated.

Tumor necrosis factor alpha (TNF-α) is a cytokine that is involved in many inflammatory and infectious diseases (1). It is secreted by activated monocytes and macrophages, which are the host cells of *T. gondii*. No protective cell effects were observed when TNF-α was added to *T. gondii*-infected mouse macrophages (2) or human fibroblasts (8). However, a synergistic effect was noted between gamma interferon (IFN-γ) and TNF-α in murine peritoneal macrophages (3). *Toxoplasma* antigens do not induce secretion of TNF-α by human mononuclear cells (12). The aim of this work was the study of TNF-α release by human monocytes and macrophages in the presence of *T. gondii* and of serum samples from individuals with high levels of specific anti-*T. gondii* antibodies. The relationship between the level of this secreted cytokine and the intracellular multiplication of the parasite in human macrophages and monocytes was evaluated.

MATERIALS AND METHODS

Monocytes. Blood was collected in tubes containing citric acid-dextrose from 11 healthy volunteers. Monocytes were isolated by a modification of the Pertoft method (10, 14). Briefly, mononuclear cells were incubated in a petri dish, and nonadherent cells were removed by washing with RPMI 1640 medium (Gibco, Cergy Pontoise, France) containing 10% AB-decomplemented (56°C, 1 h) human naive serum, without any antibodies against *T. gondii* (RPMI 10AB). The adherent cells were collected by a cell lifter (Costar, Cambridge, Mass.) and counted in a Neubauer cell. Viability was assayed by the ethidium bromide-acridine orange assay (Becton Dickinson, Oxnard, Calif.). Only monocyte preparations that were >95% viable and >95% pure were included in this study. Monocyte purity was routinely assesed by May-Grundwald-Giemsa and specific esterase staining.

Monocyte-to-macrophage maturation. Monocytes were cultured (24-well plates) in RPMI 10AB at 37°C in a 5% CO_2-enriched atmosphere for 7 days to allow monocyte-to-macrophage maturation (6). AB serum was free of antibodies to *T. gondii*, as verified by an immunofluorescence assay. The culture medium was changed every 2 days.

***T. gondii*.** The RH strain of *T. gondii* was obtained by culture in vitro in human fibroblast MRC5 cells (BioMerieux, Marcy l'Étoile, France). Tachyzoites were collected from the culture supernatant (1,200 × *g* for 10 min) after 4 days and then counted in a Neubauer cell. Viability was assayed as described above, and only parasite preparations with a viability of >95% were used.

Positive, negative, and autologous sera. Positive sera were obtained from patients with high levels of antibodies to *T. gondii* (1,280 IU/ml, indirect fluorescent-antibody technique). Negative sera, obtained from healthy people, had titers of less than 8 IU/ml. Autologous sera were obtained from the corresponding monocyte donors. All of the sera used in this study were TNF-α free.

Other positive sera. Other positive sera were kindly provided by P. Marty (Nice, France) and B. Gratacap (Grenoble, France). These sera had specific antibodies against *Amoeba*, *Candida*, *Leishmania*, *Chlamydia*, *Legionella*,

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Brucella, *Treponema*, and *Streptococcus* spp. All of them were negative for *T. gondii* antibodies and for TNF-α.

Study of TNF-α secretion. Monocytes were distributed into 24-well plates, at 2.5×10^4 cells per well, in 1 ml of RPMI 10AB and incubated for 1 h at 37°C. Then, a suspension containing 2×10^5 parasites (incubated with 100 μl of positive, negative, or autologous serum for 1 h at 37°C) was added to the wells. Positive serum (100 μl) alone was also tested. After another hour, the wells were washed with RPMI 10AB in order to remove extracellular parasites. The plates were incubated for 48 h at 37°C in a 5% CO_2 atmosphere after the cells, parasites, and serum were added. Supernatants were collected and kept at −20°C until use. Controls were obtained by lipopolysaccharide (LPS) stimulation (0.1 μg/ml; Calbiochem, Meudon, France) of monocytes and macrophages. In some experiments, polymyxin B (1 μg/ml; Pfizer, Orsay, France) was added to the wells in order to inhibit LPS action. In the other experiments, parasites were incubated with positive, negative, or autologous serum and then washed three times by centrifugation at 1,200 × *g* for 10 min. Ultracentrifugation was performed at 100,000 × *g* for 1 h at 4°C.

TNF-α assay. We used the immunoradiometric assay kit for human TNF-α (Medgenix, Brussels, Belgium). All positive, negative, and autologous sera used in this study were free of TNF-α (<6 pg/ml).

Incorporation of [^{3}H]uracil. At 24 h after infection with the parasite, 1 μCi of [5,6-^{3}H]uracil (specific activity, 48 Ci/mmol; NEN, Paris, France) was added to each well containing monocytes or macrophages (16). Monolayers were dissolved in 0.1 N NaOH, and the radioactivity was counted as indicated by Pfefferkorn et al. (15).

C_{1q}-binding test. Immunecomplexes were determined by the C_{1q}-binding test as described by Nydegger et al. (13).

IgG fractions. Immunoglobulin G (IgG) fractions were obtained by DEAE-Trisacryl chromatography (0.05 M Tris-HCl [pH 8.6], 0.15 M NaCl) as described by the manufacturer (IBF, Paris, France).

Statistical analysis. Statistical analysis was performed by the nonparametric signs test for comparison of independent means.

RESULTS

Absence of TNF-α release from cells induced by *T. gondii*. *T. gondii* tachyzoites alone do not induce secretion of TNF-α by human monocytes in vitro (Fig. 1). No significant difference in TNF-α levels was observed between supernatants from mononuclear cells stimulated by the parasite and the corresponding supernatants from unstimulated cells.

Human mononuclear cell release of TNF-α by *T. gondii* and serum. When monocytes were incubated with parasites and positive serum, secretion of TNF-α was the highest ($P < 0.05$) in comparison with secretion obtained with monocytes alone, LPS-stimulated monocytes, monocytes plus *T. gondii*, and monocytes plus *T. gondii* incubated in negative or autologous serum. Similar data were obtained with macrophages (Fig. 1), but monocytes released more TNF-α into the culture fluid ($P < 0.05$).

In six experiments, when 1,280 IU of anti-*Toxoplasma* serum per ml was added to the cells, released TNF-α levels were higher than those induced by parasites plus positive serum (Fig. 2). This inhibition of the secreted TNF-α level varied from 11.4 to 71.8%. Nevertheless, *Toxoplasma*-infected monocytes produced TNF-α when they were subsequently stimulated for 2 h with the pooled positive serum

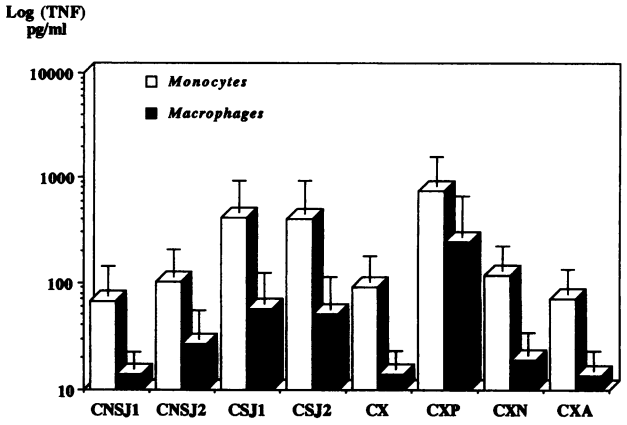

FIG. 1. TNF-α produced by monocytes or macrophages. When monocytes or macrophages were incubated with parasites and positive serum, secretion of TNF-α was the highest ($P < 0.05$) in comparison with secretion obtained with monocytes or macrophages alone, LPS-stimulated monocytes or macrophages, monocytes or macrophages plus *T. gondii*, and monocytes or macrophages plus *T. gondii* incubated in negative or autologous serum. CNSJ 1, unstimulated controls, day 1; CNSJ 2, unstimulated controls, day 2; CSJ 1, LPS-stimulated controls, day 1; CSJ 2, LPS-stimulated controls, day 2; CX, monocytes or macrophages and parasites; CXP, monocytes or macrophages, parasites, and serum (1,280 IU/ml); CXN, monocytes or macrophages, parasites, and negative serum; CXA, monocytes or macrophages, parasites, and autologous serum. Error bars represent one standard deviation.

(Fig. 2). In addition, LPS-stimulated cells in the presence of the parasite induced as much TNF-α secretion as LPS-stimulated cells alone.

In order to evaluate the induction of TNF-α secretion by the antibody-*T. gondii* complex, three different experiments were performed in which parasites were incubated in serum (1,280 IU/ml) for 1 h and then washed at 1,200 × *g* for 10 min to remove unbound components. Secreted TNF-α levels were found to be higher than in controls but lower than those obtained with the pooled positive serum (Table 1).

To estimate whether aggregated immunoglobulins were

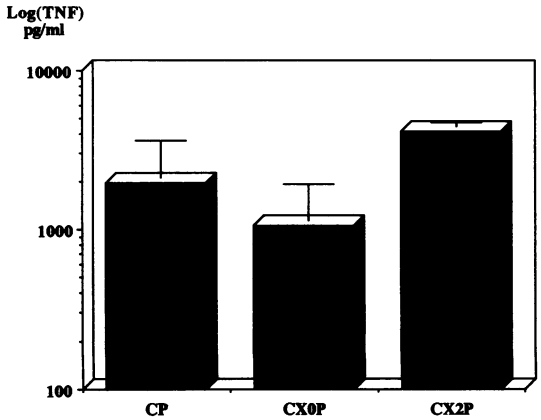

FIG. 2. Relationships between TNF-α production, *T. gondii* infection, and positive serum. The data represent the results of six experiments. CP, monocytes plus serum (1,280 IU/ml); CX0P, monocytes plus *T. gondii* plus serum (1,280 IU/ml) added at the time of infection; CX2P, monocytes plus *T. gondii* plus serum (1,280 IU/ml) added 2 h after cell infection. Error bars represent one standard deviation.

TABLE 1. TNF-α production induced by positive serum alone or by parasites incubated in serum and washed

Assay	TNF-α (pg/ml)		
	Expt 1	Expt 2	Expt 3
Positive serum alone (control)	220	151	50
Monocytes plus serum (1,280 IU/ml)	2,670	4,440	720
Monocytes plus incubated and washed parasites	1,530	902	423

able to induce TNF-α secretion, ultracentrifuged (100,000 × *g* for 1 h at 4°C) positive-serum supernatant was assayed. This procedure did not significantly decrease the ability of the serum to induce TNF-α release (720 versus 395 pg/ml).

To determine whether autoantibodies against mononuclear cells were involved, an indirect fluorescent-antibody test was performed. No autoantibodies were detected in the positive serum.

The sera which have antibodies against other pathogens but not for *T. gondii* were free of TNF-α and did not induce TNF-α release except for the three samples containing antibodies against a *Leishmania* sp. In this case, the TNF-α levels were lower than those obtained with the serum containing anti-*T. gondii* antibodies (Fig. 3).

Endotoxin contamination. To determine whether the LPS present in the positive serum induced TNF-α release, experiments with polymyxin B (1 μg) were performed (19). No significant difference in TNF-α levels was noted when polymyxin B was added to the serum.

Lack of correlation between TNF-α secretion and [^{3}H]uracil incorporation. In this study, no correlation was observed between TNF-α secretion by monocytes or macrophages and the intracellular multiplication of the parasite, as evaluated by [^{3}H]uracil incorporation (Fig. 4 and 5).

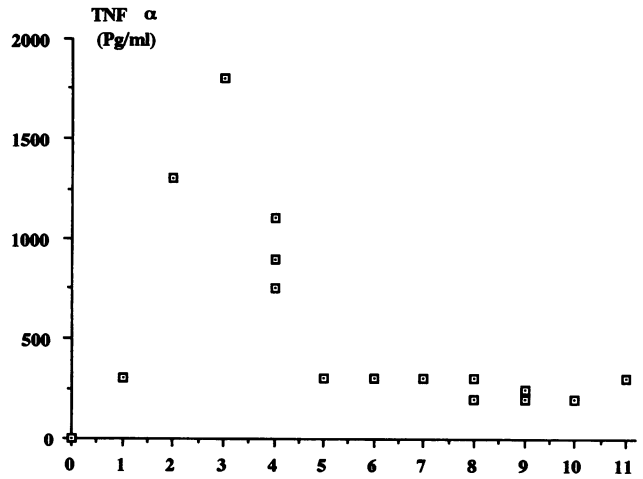

FIG. 3. TNF-α secretion induced by serum samples from patients with other infectious diseases. Only three serum samples containing antibodies against a *Leishmania* sp. induced TNF-α secretion. In this case, the TNF-α levels were lower than those obtained with the serum containing anti-*T. gondii* antibodies. Samples: 1, monocytes; 2, monocytes stimulated by LPS; 3, monocytes and anti-*T. gondii* serum (1,280 IU/ml); 4, monocytes and anti-*Leishmania* serum (three sera); 5, monocytes and anti-*Amoeba* serum; 6, monocytes and anti-*Candida* serum; 7, monocytes and anti-*Echinococcus* serum; 8, monocytes and anti-*Treponema* serum (two sera); 9, monocytes and anti-*Brucella* serum (two sera); 10, monocytes and anti-*Legionella* serum; 11, monocytes and anti-*Chlamydia* serum. Data were obtained in one experiment.

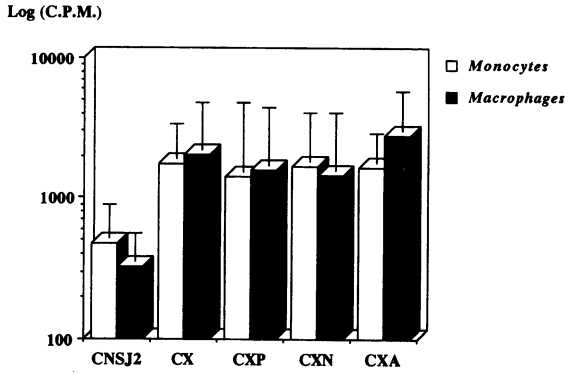

FIG. 4. Evaluation of intracellular multiplication of *T. gondii* in monocytes or macrophages by incorporation of [^{3}H]uracil. CNSJ 2, unstimulated controls, day 2; CX, monocytes or macrophages and parasites; CXP, monocytes or macrophages, parasites, and serum (1,280 IU/ml); CXN, monocytes or macrophages, parasites, and negative serum; CXA, monocytes or macrophages, parasites, and autologous serum.

DISCUSSION

The results reported here show that *T. gondii* alone does not induce secretion of TNF-α by activated human monocytes or macrophages in vitro. The absence of TNF-α induction is a reversible phenomenon, because after *Toxoplasma* invasion and addition of positive pooled serum or LPS, the infected cells are again able to secrete TNF-α. In our study, the lack of TNF-α secretion agrees with the results of a previous study (12) in which soluble *Toxoplasma* antigen stimulated human monocytes to secrete interleukin-1 but not TNF-α. However, monocytes or macrophages incubated with positive serum plus parasites can secrete this cytokine. In a recent study, it was shown that *T. gondii* tachyzoites can either actively invade the host and survive or be phagocytosed and die (11). This finding and ours may

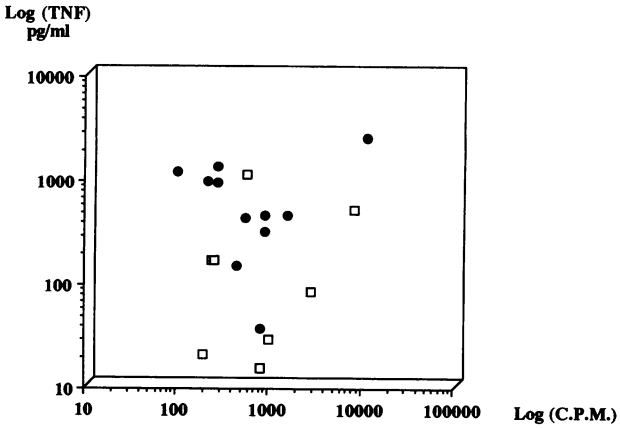

FIG. 5. Absence of relationship between TNF-α secretion by monocytes or macrophages and the intracellular multiplication of *T. gondii*. ●, monocytes; □, macrophages. Each point represents the value obtained in an individual experiment.

suggest that the absence of TNF-α induction is related to active host cell *T. gondii* invasion, which occurs in the absence of antibodies, while positive TNF-α induction is associated with parasite phagocytosis via macrophage Fc receptors. However, in our study, TNF-α levels were not related to the intracellular multiplication of the parasite as measured by [^{3}H]uracil incorporation. De Titto et al. (8) observed similar results concerning the intracellular multiplication of *T. gondii* in normal murine peritoneal macrophages and in human fibroblasts. Therefore, this cytokine does not seem to be implicated in the direct killing of the parasite by human macrophages or monocytes.

In this study, at least two factors could be responsible for the TNF-α secretion: one soluble factor that bound to human monocytes and was contained in the pooled positive-serum supernatant after parasite incubation, and one that bound to the parasites after incubation in pooled, positive, decomplemented serum.

Circulating antigens from *T. gondii* could also be suspected; however, Kelly et al. (12) have shown that soluble *Toxoplasma* antigens obtained by freeze-thaw lysis of the parasites did not induce TNF-α secretion. Thus, it is possible that circulating antigens are not able to induce secretion of this cytokine. Aggregated immunoglobulin and macrophage autoantibodies may also be involved in TNF-α release, but the results with supernatants obtained by ultracentrifugation of the positive serum and immunofluorescence showed that in our case this was not a valid hypothesis. Antibodies against the parasite may also be responsible for this secretion because of their ability to bind to parasites by the Fab component and to macrophages by the Fc moiety. In fact, DEAE fractions from Trisacryl chromatography of pooled sera showed only a slight increase in TNF-α levels. It has also been demonstrated that the Fc fragment could induce secretion of TNF-α by cross-linkage (7). In contrast, in a rabbit model, human IgG can suppress TNF-α production (18). In this study, antigen-antibody complexes which might cross-link Fc receptors were not present in pooled positive serum, as revealed by the C_{1q}-binding test.

Our control procedures showed that TNF-α (<6 pg/ml) was not detected in the positive sera from patients with toxoplasmosis who had elevated levels of IgG antibodies. Therefore, these cytokine levels could not be enough to stimulate monocyte or macrophage release. Interleukin-1 may also be involved in TNF-α release; nevertheless, Kelly et al. (12) showed that interleukin-1 secretion by *T. gondii*-infected monocytes is independent of TNF release. IFN-γ could also be suspected (4) in the activation of monocytes or macrophages and subsequent release of TNF-α. However, a recent publication noted that IFN-γ is detected in the sera of patients with acute toxoplasmosis only before the appearance of high IgG antibody levels (17). In this study, IFN-γ levels were expected to be low because only sera with high anti-*Toxoplasma* antibody titers were used. In addition, no IFN-γ was detected by enzyme-linked immunosorbent assay in the sera used. The LPS contained in serum may also be involved in TNF-α release (1), but addition of polymyxin B, an inhibitor of LPS-induced cytokine release (19), did not decrease TNF-α secretion, suggesting that this endotoxin was not present in the biological samples used. Another unidentified serum factor(s) could stimulate monocyte or macrophage TNF-α secretion. This possibility was supported in part by our experiments involving sera from patients with acute leishmaniosis that were TNF-α free and negative for antibodies to *T. gondii*. Preliminary attempts to characterize the factor(s) indicated that it survived 24 h at 4°C plus 1 h at 56°C, was probably induced by *Toxoplasma* and *Leishmania* factors, and is different from antibodies, TNF-α, interleukin-1, IFN-γ, and LPS. The activity was retained in a dialysis membrane with a 5,000-Da pore size. Further elucidation of the factor(s) responsible will provide insight into this type of immune response and its implication in T-cell activation.

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