Human Platelet-mediated Cytotoxicity against *Toxoplasma gondii*: Role of Thromboxane

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Summary

Human platelets, in the absence of antibody, are cytotoxic to tachyzoites of *Toxoplasma gondii* as determined by vital staining, transmission electron microscopy, and the failure of *Toxoplasma* to survive and replicate in mice after in vitro interaction of the organisms with platelets. Platelet to *T. gondii* ratios as low as 1:3 were toxic to the organisms with direct cell–cell contact essential for platelet-mediated cytotoxicity. Adherence of platelets to *T. gondii* and disruption of surface membranes and cytoplasmic contents of the organisms were observed ultrastructurally. Reactive oxygen species were not implicated in the platelet-mediated toxicity. The interaction of *T. gondii* with platelets resulted in a marked increase in thromboxane B_2 (TXB_2) production compared with that by unstimulated platelets. The cyclooxygenase inhibitors acetylsalicylic acid and indomethacin inhibited platelet-mediated cytolytic activity as did the selective TXA_2 synthetase inhibitor dazmegrel, indicating a role for thromboxane in the platelet-induced cytotoxicity. Further, toxoplasmacidal activity was retained in the TXA_2 synthetase-containing microsomal fractions of platelets disrupted by freezing and thawing; cytolytic activity was absent in microsome-depleted platelet supernatant fractions. Both the TXA_2-generating platelet microsome system and a stable TXA_2 analogue induced damage to the cellular membranes of the *Toxoplasma* as noted by transmission electron microscopy. These findings suggest that platelets may play a role in the host defense against *Toxoplasma* and that release of thromboxane may be important in this cytolytic process.

Toxoplasma gondii is an obligate intracellular protozoan that actively invades macrophages without triggering respiratory burst activity, prevents phagolysosomal fusion, and replicates intracellularly to the detriment of the host (1–3). Toxoplasmosis, the disease caused by this intracellular coccidian parasite, is of increasing concern because of its high incidence in immunocompromised patients (4). Toxoplasmic encephalitis is the most common cause of focal central nervous system lesions in patients with AIDS (5). Parasitemia can be demonstrated in acute infections (6, 7) and in reactivation toxoplasmosis in immunocompromised patients (8, 9). Neutrophils (10), monocytes (10, 11), and cytokine (e.g., IFN-γ)-activated macrophages (11, 12) have cytotoxic activity against these organisms and contribute toward control of infection.

Recent attention has been directed to the cytotoxic activity of platelets against various target cells. Platelet-induced antibody-dependent cell-mediated cytotoxicity (ADCC)[1] against antibody-sensitized sheep (13, 14) and human (15) erythrocytes, schistosomula of *Schistosoma mansoni* (16–18), and microfilariae of *Brugia malayi* (19) has been demonstrated in vitro. Platelet-mediated ADCC activity resides preformed in platelet membranes and may involve phospholipase A_2 reaction products (20). Whereas mouse platelet-mediated cytotoxicity against erythrocytes is dependent upon C3 and IgG immunoglobulins (13, 14), human platelet-mediated cytotoxicity against *S. mansoni* (16), and *B. malayi* (19) is dependent upon IgE. In addition, IFN-γ (18) and C-reactive protein (17) can each promote platelet killing of schistosomula. Human platelets are toxic to certain adherent human tumor cell lines in the absence of antibody (21). This tumoricidal activity is blocked by inhibitors of arachidonic acid metabolism (21). Similarly, phospholipase A_2 reaction products have been implicated in NK cell–mediated target cell lysis (22–24).

We report here that human platelets are toxic to *T. gondii* tachyzoites in the absence of added antibody. Platelet adherence to the surface of the parasites and disruption of the surface membranes and internal architecture of the *Toxoplasma* were observed. Further, our results suggest a prominent role for the cyclooxygenase arachidonate metabolite thromboxane (TX) A_2 in this platelet-mediated toxoplasmacidal activity.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; AO, acridine orange; CGD, chronic granulomatous disease; CTA_2, carbocyclic thromboxane A_2; EB, ethidium bromide; IFA, indirect fluorescent antibody; O_2^-, superoxide anion; PD, phosphate-buffered saline, Ca^{2+}/Mg^{2+}-free; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TX, thromboxane.

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Materials and Methods

Special Reagents. PG H_2 and carbocyclic thromboxane A_2 (CTA_2) were obtained from Cayman Chemical Co. (Ann Arbor, MI); human thrombin and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), dihydrochloride from Calbiochem-Behring Corp. (La Jolla, CA); acridine orange (AO) from Allied-Signal Corp. (Morris Township, NJ); acetylsalicylic acid, ethidium bromide (EB), indomethacin, ferricytochrome *c*, superoxide dismutase (2,500 U/mg), and EDTA, dipotassium salt from Sigma Chemical Co. (St. Louis, MO); and glutaraldehyde from Polysciences, Inc. (Warrington, PA). Dulbecco's PBS (0.14 M NaCl, 2.7×10^{-3} M KCl, 8.1×10^{-3} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 9.0×10^{-4} M $CaCl_2$, and 4.9×10^{-4} M $MgCl_2$, pH 7.2) and Ca^{2+}/Mg^{2+}-free PBS (PD) were obtained from Gibco Laboratories (Grand Island, NY). Indomethacin was dissolved in 0.1 ml Tris buffer, pH 8.1, at a concentration of 10^{-2} M and diluted with PBS. The selective TX synthetase inhibitor, UK-38,485 (dazmegrel; 3-(1*H*-imidazol-1-ylmethyl)-2-methyl-1*H*-indole-1-propanoic acid) (25) was kindly provided by Dr. P.R. Urquilla (Pfizer, Inc., Groton, CT). Dazmegrel was dissolved in 0.1 N NaOH in PBS at a concentration of 10^{-2} M with dilutions made in PBS. The final pH of the indomethacin and dazmegrel solutions was 7.0.

Toxoplasma gondii. *T. gondii* RH strain was kindly provided by Dr. C.B. Wilson (University of Washington). The *Toxoplasma* were maintained by i.p. passage in BALB/c mice as previously described (26). Organisms were harvested by peritoneal lavage using PD, separated from leukocytes by filtration through a 3-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA), and centrifuged at 1,000 *g* for 15 min at 4°C. The *Toxoplasma* were washed twice by sequential resuspension in PD and centrifugation at 1,000 *g* for 15 min at 4°C. After counting in a hemocytometer, the *Toxoplasma* were resuspended in PBS. A clinical isolate of *T. gondii* (ER strain) was isolated from the brain biopsy of an adult male AIDS patient who had encephalitis. The ER strain *Toxoplasma* were propagated in human embryonic tonsil fibroblasts, which were a generous gift of Dr. Lawrence Corey (Children's Hospital and Medical Center, Seattle, WA). After washing in PD, the ER strain *Toxoplasma* were resuspended in PBS. Both the RH and ER strains of *Toxoplasma* were >95% viable when assessed by trypan blue dye exclusion (27).

Human Subjects. Platelets were isolated from the blood of 24 normal human volunteers and 1 patient with chronic granulomatous disease (CGD). In some studies, volunteers ingested 1.3 mg/kg of acetylsalicylic acid 2 h before blood collection to block platelet formation of cyclooxygenase arachidonate products (28). Otherwise, the subjects had not taken acetylsalicylic acid or other nonsteroidal antiinflammatory drugs for 2 wk before the study.

Toxoplasma-specific IgG and IgM Antibody Tests. Each blood donor was negative for *Toxoplasma*-specific IgG and IgM antibodies when tested with the indirect fluorescent antibody (IFA) technique performed according to the manufacturer's recommendations (Microbiological Research Corp., Bountiful, UT). Briefly, these tests use RH strain *Toxoplasma* as the antigen substrate that is dried and fixed on microscope slides. After incubation with test serum, the slides are rinsed and either fluorescein-conjugated anti-human IgG (γ chain specific) or fluorescein-conjugated IgM (μ chain specific) is applied. After a second incubation, the slide is again rinsed and examined under a fluorescence microscope for the typical peripheral staining reaction. The lack of fluorescence at a 1:16 serum IgG-IFA titer and at a 1:8 serum IgM-IFA titer are considered negative, indicating the lack of prior infection (either acute or chronic) with *T. gondii*.

Platelet Isolation. For platelet isolation, venous blood was collected in K_2EDTA (0.2 ml of 10% K_2 EDTA in 10 ml of blood), diluted 1:1 (vol/vol) with PD and centrifuged at 200 *g* for 15 min at 4°C. The platelet-rich plasma was collected and then centrifuged at 1,000 *g* for 15 min at 4°C. The resulting platelet pellet was washed twice by sequential resuspension in PD and centrifugation at 1,000 *g* for 15 min at 4°C. After counting in a hemocytometer, the platelets were resuspended in PBS and used immediately. The preparations contained <0.05% contamination with nucleated cells.

Platelet–T. gondii Interactions. Duplicate samples of *Toxoplasma* (10^7 organisms/ml) were added to platelets and other components of the reaction mixtures at the concentrations indicated in the legends to the figures and tables in polystyrene tubes (12 × 75 mm) in an oscillating water bath at 37°C for 90 min. Duplicate 10-μl samples were taken from each experimental condition after 0-, 60-, 90-min incubations of platelets or disrupted platelet fractions (see below) with *T. gondii* for assay of viability of the organisms by trypan blue staining and AO/EB fluorescence microscopy. After 90 min of incubation, the reaction mixtures were centrifuged at 1,000 *g* for 15 min at 4°C and the supernatants collected and stored at −70°C until RIAs for TXB_2, PGE_2, and 6-keto-$PGF_{1\alpha}$ were performed.

For studies to assess the effect of antibody on the platelet–*Toxoplasma* interactions, immune sera were obtained from (*a*) an asymptomatic individual with an IgG-IFA titer of 1:2,048 and a negative IgM-IFA titer < 1:8 and (*b*) a patient with acute toxoplasmosis who had an IgG-IFA titer of 1:1,024 and an IgM-IFA titer of 1:512. Normal serum was obtained from an individual who lacked *Toxoplasma*-specific antibodies (i.e., IgG-IFA < 1:16 and IgM-IFA < 1:8). Fresh normal sera and normal and immune sera that had been heat-inactivated at 56°C for 45 min were used at a 1:10 dilution (final concentration) in the platelet–*Toxoplasma* interactions.

To assess the in vivo survival of *Toxoplasma* that had been incubated with platelets in vitro, 1-ml reaction mixtures containing 10^7 *T. gondii* that had been incubated in the absence or presence of 10^8 platelets for 90 min at 37°C were injected into duplicate pairs of BALB/c mice. Animals were examined on a daily basis for survival. Mice still living 14 d after injection were classified as survivors and sacrificed to assess i.p. infection with *Toxoplasma*.

In some experiments, disrupted platelet fractions were substituted for intact platelets in the reactions with *T. gondii*. Platelets (2×10^8) in 1 ml were degranulated by treatment with 5 U/ml human thrombin for 15 min at 37°C (29) with the reaction terminated by the addition of 10^{-6} M PPACK (30). Platelets (6×10^8) in 3 ml of PBS were also disrupted by three cycles of freezing at −70°C for 15 min followed by thawing in a 37°C shaking water bath for 15 min over a 90-min period as previously described (14). The entire contents of the frozen and thawed platelets were used in some experiments or comparably disrupted samples were centrifuged at 5,000 *g* for 15 min at 4°C for removal of cellular debris. The supernatant was centrifuged at 100,000 *g* for 60 min at 4°C as previously described (31, 32) to obtain a microsome-depleted supernatant and a microsome-containing particulate fraction which was suspended in 3 ml PBS.

In other studies, intact platelets and *T. gondii* were cocultured in individual chambers separated by a 0.4-μm polycarbonate membrane in a 6-well (24-mm-diam) Transwell plate (Costar Data Packaging Corp., Cambridge, MA). *Toxoplasma* (2×10^7) were added to 2 ml PBS in the bottom chamber and platelets (2×10^7) in 2 ml PBS were placed in the upper chamber. The plates were centrifuged at 1,000 *g* for 30 min at 20°C to facilitate exchange of media between the chambers and then incubated an additional 60 min at 37°C before assay of *T. gondii* viability.

Assay of T. gondii Viability. Viability of *T. gondii* was determined

by trypan blue staining (27) and fluorescence microscopy. Differential fluorescence of viable and nonviable *T. gondii* was examined by reaction with AO and EB as previously described (33). Each sample was mixed with an equal volume of an AO (2.5 μg/ml)/EB (5 μg/ml) solution before placement on a glass slide in preparation for fluorescence microscopy using an Olympus Vanox microscope with epiilluminator (Olympus Corporation of America, New Hyde Park, NY). *Toxoplasma* were counted at 200× magnification using a micrometer disc (Bausch & Lomb Inc., Rochester, NY) containing 64 grids (total grid area = 2,000 μm^2); 10 random fields were selected for each sample. Viable organisms demonstrated a bright green nuclear fluorescence, whereas nonviable *Toxoplasma* were orange.

Transmission Electron Microscopy. Pellets of 2 × 10^7 *T. gondii* incubated with the various components of the reaction mixture (see legends to figures) were collected by centrifugation, fixed in 2% glutaraldehyde, and prepared for transmission electron microscopy as previously described (34). The samples were examined with a JEOL 100 B electron microscope (JEOL USA, Electron Optics Division, Medford, MA) at 60 kV.

Superoxide Anion Assay. Superoxide anion (O_2^-) generation was measured by the capacity of O_2^- to reduce ferricytochrome *c* to ferrocytochrome *c* in a microassay system as previously described (35). In brief, the 0.2-ml reaction mixture consisted of PBS, 8% albumin, 54.5 μM ferricytochrome *c*, 10^7 platelets, and the presence or absence of 10^6 *T. gondii*. The reactions were performed in quadruplicate wells of 96-well microtiter plates (Costar Data Packaging Corp., Cambridge, MA) in the presence or absence of 62.5 μg/ml superoxide dismutase for 90 min at 37°C. Nanomoles of ferricytochrome *c* reduced were calculated from the maximal increase in absorbance measured at 550 nm in a microtiter plate reader (model 309; Bio-Tek Instruments, Inc., Burlington, VT).

RIAs. PGE_2 and the respective stable hydrolysis products of PGI_2 and TXA_2, 6-keto-$PGF_{1\alpha}$ and TXB_2, were assayed by RIA. The PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2 antisera were produced in rabbits in our laboratory and their cross-reactivities have been reported (36, 37). The PGE_2 antiserum at a dilution of 1:6,000 had a sensitivity of 10 pg per 0.1-ml sample, the 6-keto-$PGF_{1\alpha}$ antiserum at a 1:6,000 dilution had a sensitivity of 10 pg per 0.1-ml sample, and the TXB_2 antiserum at a dilution of 1:100,000 had a sensitivity of 1 pg per 0.1-ml sample. Synthetic standards were the generous gift of Douglas McCarter (Upjohn Co., Kalamazoo, MI). Labeled tracers ([^{3}H]PGE_2, [^{3}H]6-keto-$PGF_{1\alpha}$, and [^{3}H]TXB_2) were obtained from New England Nuclear (Boston, MA) and unlabeled synthetic prostanoid standards were obtained from Cayman Chemical Co. Each assay was performed in duplicate according to standard protocols.

Statistical Analysis. The data are reported as the mean ± SE of the combined experiments. Differences were analyzed for significance ($p < 0.05$) by Student's two-tailed *t* test for independent means.

Results

Platelet-mediated Cytotoxicity against T. gondii. Incubation of *T. gondii* (RH strain) for 90 min at pH 7.0 with intact human platelets in the absence of serum resulted in a significant increase in nonviability of the *Toxoplasma* to 18.9% from a background of 6.4% under the conditions used in Table 1. The platelet (effector)/*Toxoplasma* (target) cell ratio was 1:3. Toxicity as assessed by trypan blue staining and by orange fluorescence of the organisms upon vital staining with AO/EB increased progressively with higher ratios of platelets to *Toxoplasma* up to 84.7% nonviability of the organisms at a 100:1 ratio (Table 1). Human platelets had a similar cytotoxic effect against a *T. gondii* strain that had been isolated from a patient with AIDS; 60.3, 37.0, and 18.9% of these organisms were

Table 1. *Toxoplasmacidal Activity of Human Platelets*

Supplements		Nonviable *T. gondii* 0 min	60 min		90 min	
		%	%	*p*	%	*p*
Toxo (RH)		3.7 ± 0.8 (8)*	6.3 ± 1.5 (8)		6.4 ± 1.5 (8)	
Platelets + Toxo (RH)	(1:3)	4.0 ± 0.8 (8)	12.5 ± 1.5 (8)	<0.02	18.9 ± 1.6 (8)	<0.001
	(1:1)	3.2 ± 1.5 (3)	26.4 ± 2.6 (3)	<0.001	40.5 ± 8.2 (3)	<0.001
	(10:1)	3.1 ± 1.8 (3)	34.6 ± 2.9 (3)	<0.001	60.6 ± 9.1 (3)	<0.001
	(100:1)	3.6 ± 2.0 (3)	65.6 ± 3.8 (3)	<0.001	84.7 ± 0.3 (3)	<0.001
Toxo (ER)		3.6 ± 0.3 (3)	4.1 ± 0.9 (3)		7.5 ± 1.5 (3)	
Platelets + Toxo (ER)	(1:3)	5.3 ± 1.2 (3)	16.6 ± 2.6 (3)	<0.02	18.9 ± 1.3 (3)	<0.01
	(1:1)	5.4 ± 1.1 (3)	29.1 ± 5.2 (3)	<0.01	37.0 ± 2.5 (3)	<0.001
	(10:1)	3.8 ± 1.6 (3)	42.9 ± 1.3 (3)	<0.001	60.3 ± 5.3 (3)	<0.001

The reaction mixture contained 2 × 10^7 *T. gondii* (*Toxo*; RH strain [*RH*] or clinical isolate ER strain [ER]) alone or with 0.67 × 10^7 to 2 × 10^9 human platelets as indicated in a total volume of 2 ml PBS. Viability of *T. gondii* was assessed by trypan blue staining and confirmed by AO/EB fluorescence microscopy after 0-, 60-, or 90-min incubations at 37°C.

* Mean ± SE of experiments (*n*). Probability (*p*) values for the difference from *T. gondii* (RH strain or clinical isolate) alone are shown where significant ($p < 0.05$).

nonviable after 90 min of incubation of platelets with *Toxoplasma* at ratios of 10:1, 1:1, and 1:3, respectively (Table 1).

Platelet-mediated toxoplasmacidal activity was not affected by the presence of either heat-inactivated (Table 2) or fresh (data not shown) normal serum that was negative for *Toxoplasma*-specific IgG and IgM antibodies. Further, heat-inactivated immune sera that contained *Toxoplasma*-specific IgG antibodies in either the absence or presence of *Toxoplasma*-specific IgM antibodies did not alter platelet-induced cytotoxicity against *T. gondii* (Table 2). Fresh immune serum was not used since, as reported by other investigators, *Toxoplasma*-specific antibody in the presence of complement has a cytotoxic effect against the organisms (38–41).

To assess whether the demonstrated in vitro cytotoxic effect of platelets against *Toxoplasma* corresponded to decreased viability of the organisms in vivo, *T. gondii* alone and platelet–*T. gondii* (10:1 ratio) reaction mixtures (each containing 10^7 organisms) after in vitro incubation for 90 min at 37°C were injected intraperitoneally into BALB/c mice; the animals were followed for survival and recovery of viable organisms over a 2-wk study period. All control animals injected with *T. gondii* alone (n = 8) died within 5 d after injection. In contrast, 100% of the animals receiving the platelet–*T. gondii* suspensions (n = 8) survived the 14-d study period. *Toxoplasma* were recovered at necroscopy from the peritoneal fluid of each of the mice that had received *T. gondii* alone but were absent in the peritoneal fluid of the survivors that were sacrificed on day 14.

Direct cell–cell contact was essential for platelet-mediated toxoplasmacidal activity. Separation of platelets from *Toxoplasma* by a 0.4-μm polycarbonate membrane barrier in a tissue culture well blocked in vitro platelet-mediated toxoplasmacidal activity (Fig. 1).

Platelet-mediated cytotoxicity against *T. gondii* was confirmed by transmission electron microscopy. Greater than 90% of *Toxoplasma* incubated in PBS for 90 min exhibited the usual morphology of viable tachyzoites (42). The crescent shaped *T. gondii* have a continuous outer surface membrane (Fig. 2

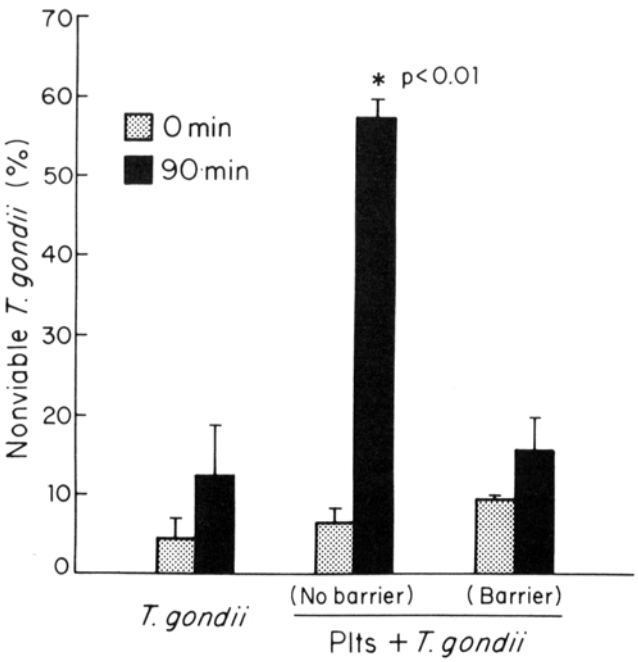

Figure 1. Effect of a membrane barrier on platelet-mediated cytotoxicity against *T. gondii*. 2 × 10^7 *T. gondii* were incubated in tissue culture wells alone or with 2 × 10^7 platelets (*Plts*) in the absence or presence of a 0.4-μm polycarbonate membrane barrier for 90 min at 37°C. Viability of the organisms was determined as described in Table 1. The data represent the mean ± SE of three experiments. Probability values for the difference from *T. gondii* alone are shown where significant ($p < 0.05$).

Table 2. *Effect of Toxoplasma-specific Antibodies on Platelet-mediated Toxoplasmacidal Activity*

Supplements	Nonviable *T. gondii* 0 min	Nonviable *T. gondii* 90 min	p
	%	%	
Toxo	3.0 ± 1.1*	6.9 ± 0.6	
+ normal serum (IgG^-/IgM^-)	4.7 ± 1.8	6.9 ± 0.1	
+ immune serum (IgG^+/IgM^-)	4.4 ± 1.4	10.8 ± 2.4	
+ immune serum (IgG^+/IgM^+)	5.5 ± 1.3	10.6 ± 1.4	
Platelets + Toxo	4.5 ± 1.6	44.5 ± 7.6	<0.01
+ normal serum (IgG^-/IgM^-)	5.7 ± 2.3	45.5 ± 2.0	<0.001
+ immune serum (IgG^+/IgM^-)	4.9 ± 1.2	42.4 ± 3.0	<0.001
+ immune serum (IgG^+/IgM^+)	4.9 ± 1.3	44.9 ± 8.2	<0.01

The reaction mixtures contained 2 × 10^7 *T. gondii* (*Toxo*; RH strain) in the absence or presence of 2 × 10^7 platelets in 2 ml PBS. In some studies as described in Materials and Methods, normal serum (IgG-IFA <1:16/IgM-IFA <1:8 [IgG^-/IgM^-]) or immune serum (IgG-IFA = 1:2,048/IgM-IFA <1:8 [IgG^+/IgM^-] or IgG-IFA = 1:1,024/IgM-IFA = 1:512 [IgG^+/IgM^+]) that had been previously heat inactivated at 56°C for 45 min was included at a 1:10 dilution (final concentration). The reaction mixtures were incubated for 90 min at 37°C with parasite viability determined as described in Table 1.

* Mean ± SE of three experiments. Probability (p) values for the difference from *T. gondii* alone are shown where significant ($p < 0.05$).

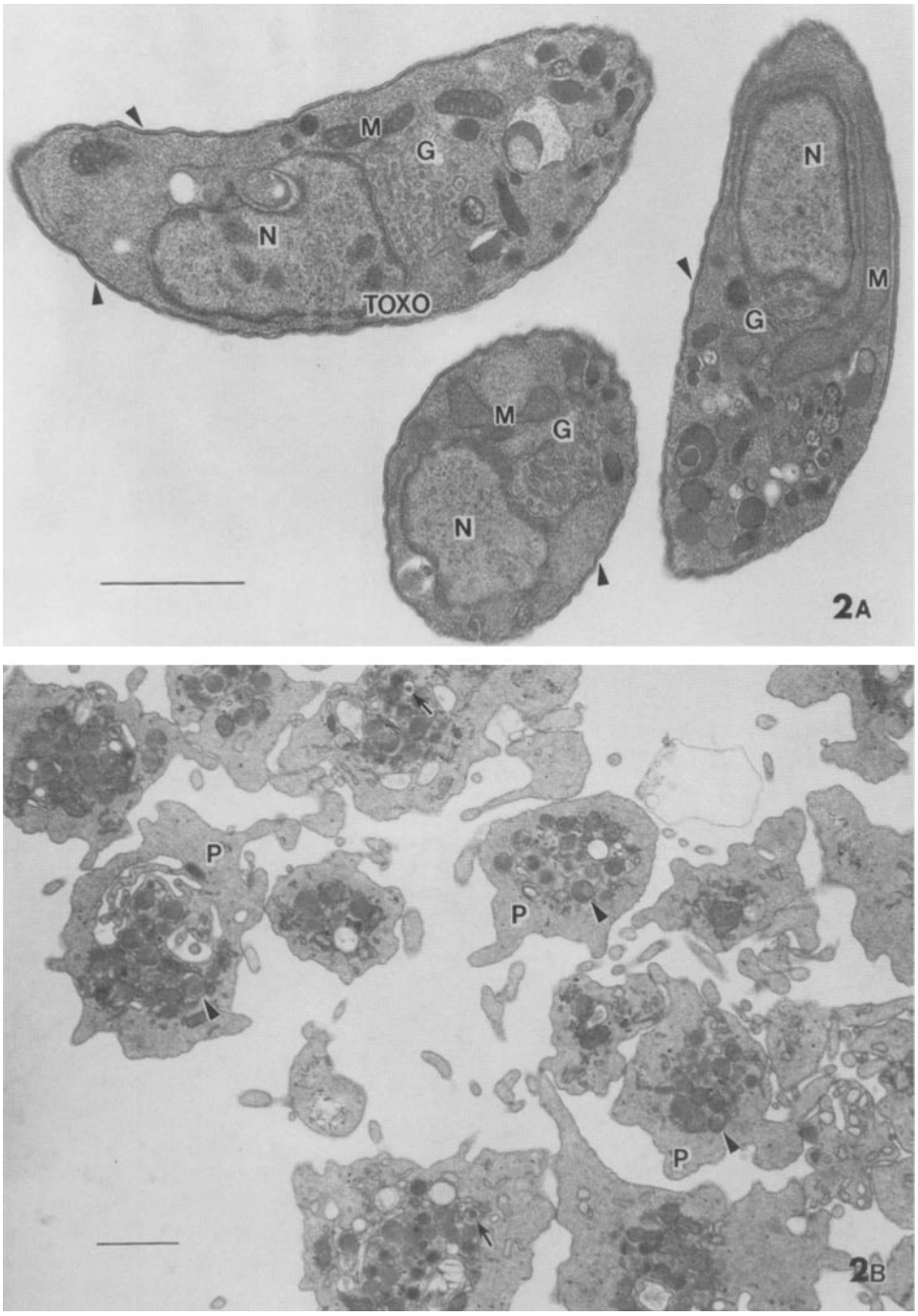

Figure 2. Ultrastructure of *T. gondii* and platelets. (*A*) *Toxoplasma gondii* were incubated in the standard salt solution for 90 min at 37°C as described in Table 1. The normal appearance of the *Toxoplasma* (*TOXO*) with their elongate and crescent appearance is shown. The usual array of cytoplasmic organelles is seen including Golgi apparatus (*G*), mitochondria (*M*), and nucleus (*N*). The surface membrane of the *Toxoplasma* consists of a characteristic double structure with inner and outer membrane units (*arrowheads*). Bar, 1 μm. ×24,000. (*B*) Purified human platelets were incubated in buffer for 90 min at 37°C. Pseudopodal surface projections are evident; the platelets (*P*) contain both α granules (*arrowheads*) and dense granules (*arrows*) in their cytoplasm; platelet aggregation was not evident. Bar, 1 μm. ×12,000.

A) beneath which is located an inner membranous layer that is interrupted at the anterior and posterior ends of the organisms. The nucleus and intracellular organelles such as Golgi apparatus, mitochondria, and endoplasmic reticulum of the *T. gondii* appeared normal in the control *Toxoplasma* (Fig. 2 *A*). The human platelets also exhibited their typical morphology (43) when incubated in PBS for 90 min. Smaller than *Toxoplasma*, platelets have a single unit plasma membrane and cytoplasmic granule heterogeneity with both α and dense granules present (Fig. 2 *B*). The control platelets had numerous surface projections, and aggregation was not evident (Fig. 2 *B*).

When platelets were incubated with *T. gondii* at a ratio of 10:1 for 30 min, circumferential attachment of the platelets by surface projections to the outer membrane of the parasites was observed (Fig. 3, *A* and *B*). The parasites exhibited marked cytotoxic changes as a consequence of this interaction with human platelets for 90 min (Fig. 4, *A* and *B*). Swelling and disruption of the parasite surface membrane structure was noted (Fig. 4, *A* and *B*); the cytoplasm of the *Toxoplasma*

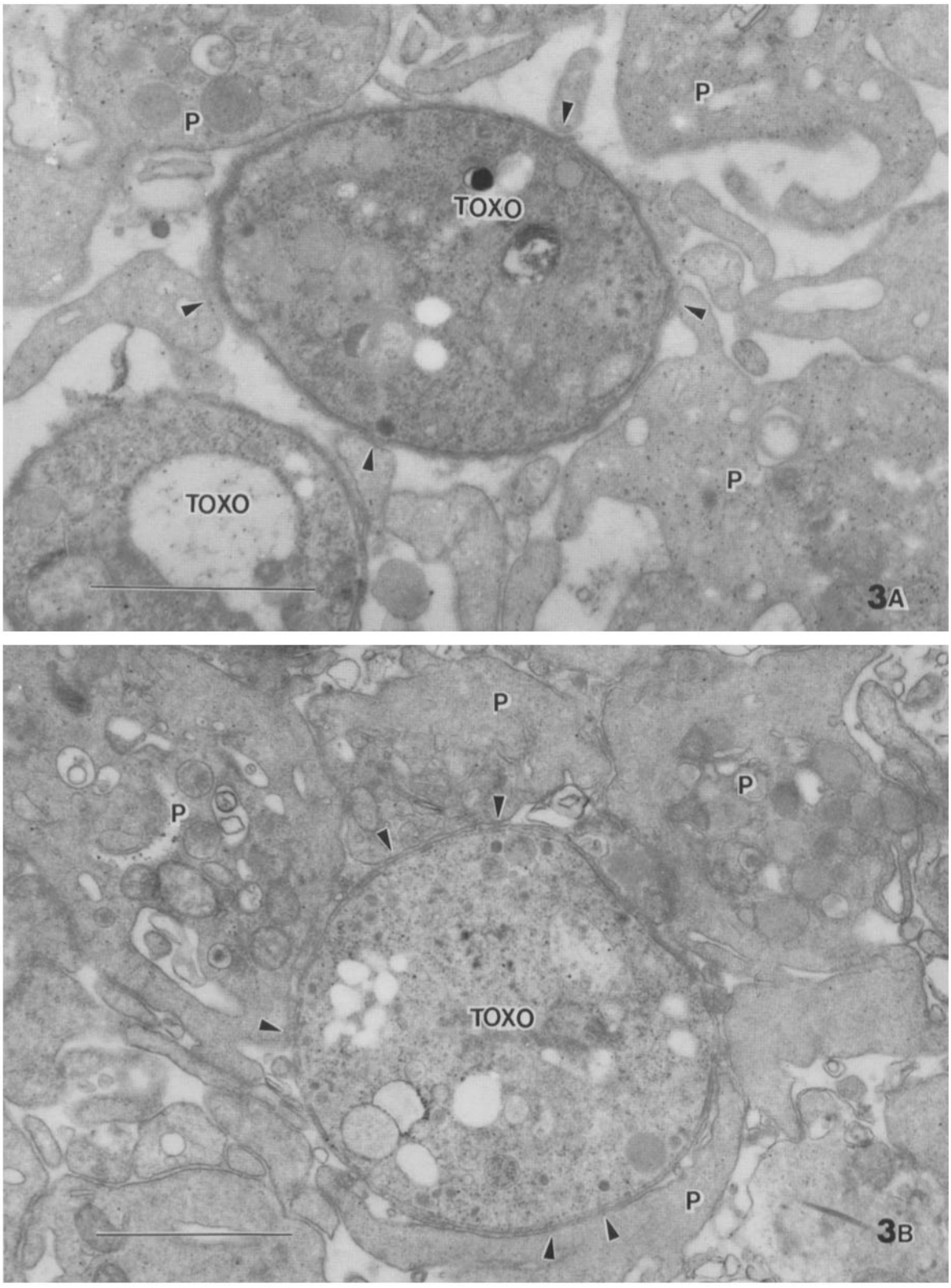

Figure 3. Adherence of platelets to *Toxoplasma*. (*A*) Human platelets were incubated with *Toxoplasma* in a ratio of 10:1 for 30 min as described in Table 1. Close apposition of platelet (*P*) pseudopodal projections (*arrowheads*) to the surface of the *Toxoplasma* (*TOXO*) is seen. Bar, 1 μm. ×40,000. (*B*) Platelet (*P*) adherence (*arrowheads*) to the surface membrane of the *Toxoplasma* was often extensive Bar, 1 μm. ×35,000.

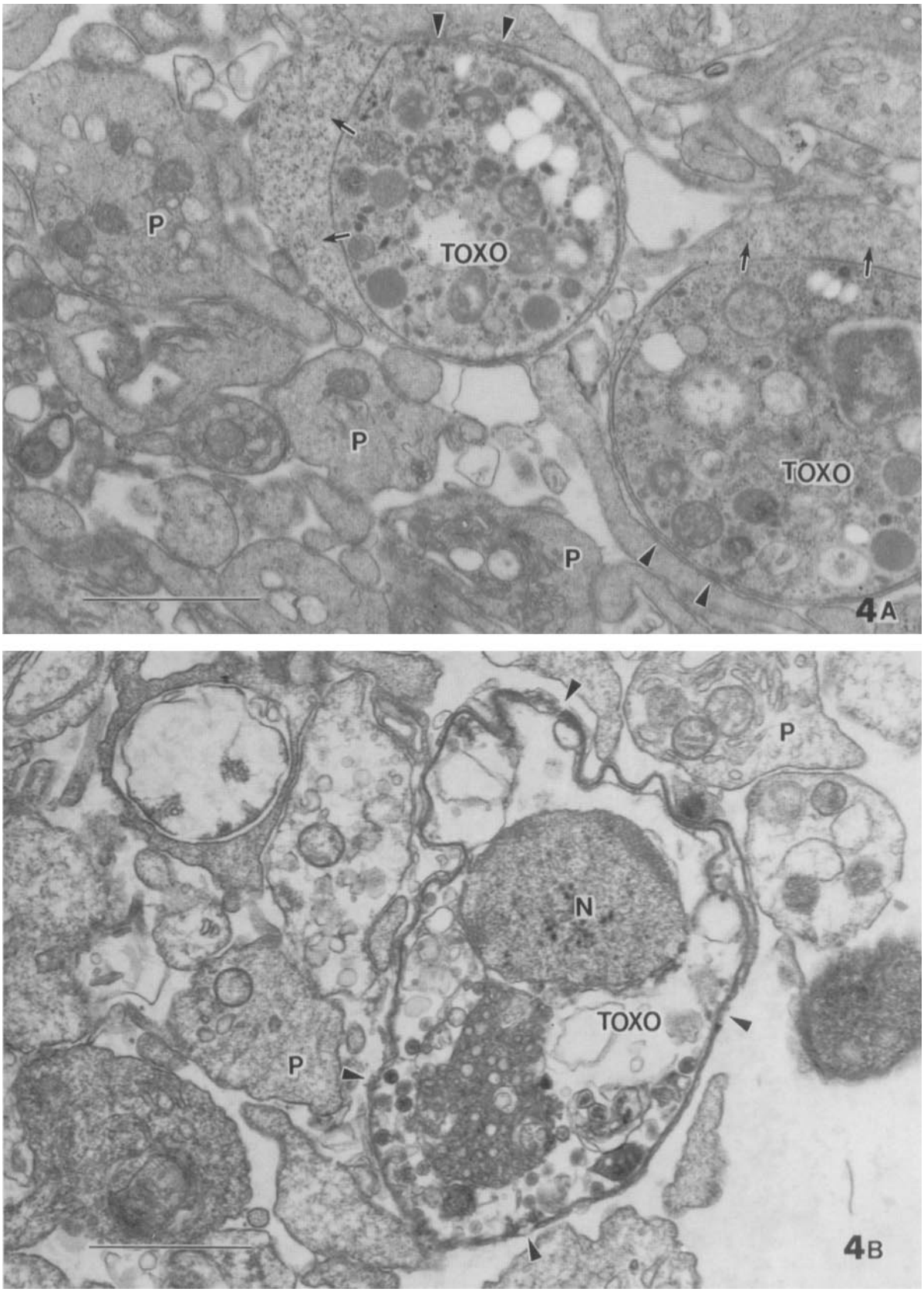

Figure 4. Platelet-induced *Toxoplasma* cytotoxicity. (*A*) Platelets and *Toxoplasma* (10:1 ratio) were incubated for 90 min as described in Table 1. Platelet (*P*) aggregation and degranulation are evident with attachment (*arrowheads*) of platelet surface projections to the surface of the *Toxoplasma* (*TOXO*). Release of cytoplasmic contents (*arrows*) through the inner parasite membrane is associated with prominent bulging of the outer parasite membrane. Bar, 1 μm. ×30,000. (*B*) Additional cytotoxic changes noted in the *Toxoplasma* (*TOXO*) after their interaction with human platelets (*P*) include disruption of the surface membrane (*arrowheads*), swelling of the membrane surrounding the nucleus (*N*), and intracytoplasmic vacuolization with disappearance of intracellular organelles (mitochondria, Golgi apparatus, and endoplasmic reticulum). Bar, 1 μm. ×28,000.

became vacuolated with loss of organelles (Fig. 4 *B*). Other cytotoxic changes noted in the *T. gondii* were dilation of their nuclear envelope membranes and disruption of their inner surface membranes; platelet aggregation was prominent in the platelet-*T. gondii* reaction mixtures.

Lack of Role of Reactive Oxygen Species in Cytotoxicity. Studies were conducted to determine the biochemical mechanism(s) of platelet cytotoxicity against *T. gondii*. Production of small amounts of chemiluminescence (44, 45) and O_2^- (46) by human platelets has been reported, and the possible contribution of platelet generation of reactive oxygen species in killing of *Toxoplasma* was examined. Human platelets, however, failed to reduce ferricytochrome *c* after incubation with *T. gondii* (10:1 ratio) for 90 min at 37°C ($n = 3$) indicating a lack of O_2^- formation during this interaction. In addition, platelets from a CGD patient effectively killed *Toxoplasma* at platelet/*T. gondii* ratios from 1:1 to 100:1 at 60 and 90 min of incubation (Fig. 5).

Release of Cyclooxygenase Arachidonate Products during Platelet-T. gondii Interaction. The possible role of cyclooxygenase products of arachidonic acid metabolism in the mediation of platelet cytotoxicity against *T. gondii* was studied. As seen in Fig. 6, human platelets incubated in buffer alone for 90 min released 34.8 pg TXB_2 and 19.4 pg PGE_2 per 10^6 platelets. PGE_2 (73.4 pg/10^6 organisms) was the predominant cyclooxygenase arachidonate product released by *T. gondii* incubated in buffer alone for 90 min. When platelets were incubated with *Toxoplasma* (1:1 ratio), TXB_2 production was augmented 3.9-fold (to a concentration of 4×10^{-9} M) compared to TXB_2 release by platelets alone ($p < 0.02$); PGE_2 release was not significantly altered from that of *T. gondii* alone. The cyclooxygenase inhibitor indomethacin (10^{-5} M) inhibited the release of TXB_2 and PGE_2 by the platelet-*Toxoplasma* reaction mixtures by 89.0 and 72.6%, respectively. Dazmegrel, a potent inhibitor of TXA_2 synthetase, has no significant activity against either cyclooxygenase or PGI_2 synthetase enzymes (25). Dazmegrel (10^{-5} M) inhibited TXB_2 release by 85.1% and stimulated PGE_2 release by 65.2% in the platelet-*T. gondii* reaction mixtures. Platelets were also isolated from individuals who had taken acetylsalicylic acid (1.3 mg/kg) 2 h before blood collection for in vivo inhibition of cyclooxygenase activity (28). After interaction with *Toxoplasma*, these platelets released < 7 pg of either TXB_2 or PGE_2 per 0.1-ml sample containing 10^6 platelets and 10^6 *T. gondii*. Less than 20 pg of 6-keto-$PGF_{1\alpha}$ were released per 0.1-ml sample containing 10^6 platelets and/or *Toxoplasma* (data not shown).

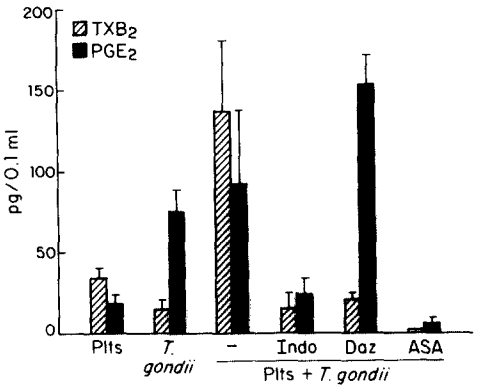

Figure 6. Release of TXB_2 and PGE_2 by platelet-*T. gondii* reaction mixtures. Supernatants were obtained from the following reaction mixtures that were incubated in a final volume of 2 ml PBS for 90 min at 37°C: 2×10^7 platelets (*Plts*) ($n = 12$); 2×10^7 *T. gondii* ($n = 18$); 2×10^7 platelets and 2×10^7 *T. gondii* in the absence ($n = 8$) or presence of either 10^{-5} M indomethacin (*Indo*) ($n = 5$) or 10^{-5} M dazmegrel (*Daz*) ($n = 3$); and 2×10^7 platelets that had been obtained from subjects who had ingested 1.3 mg/kg of acetylsalicylic acid (*ASA*) 2 h before blood collection and 2×10^7 *T. gondii* ($n = 4$). The supernatants were assayed in 0.1-ml aliquots for TXB_2 and PGE_2 by RIA.

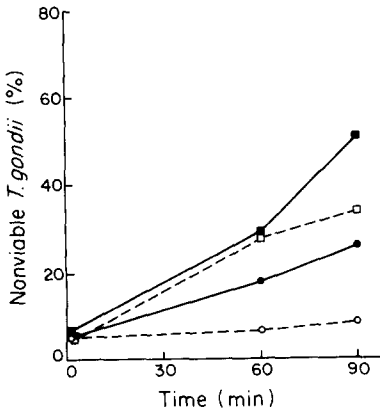

Figure 5. Toxoplasmacidal activity of CGD platelets. Platelets from a patient with CGD were incubated with *T. gondii* in PBS for 90 min at 37°C at the following ratios of platelets to *Toxoplasma*: 1:10 (○); 1:1 (●); 10:1 (□); and 100:1 (■). Viability of the organisms was assessed by trypan blue staining. The data are the mean of two experiments using platelets from a single patient with CGD.

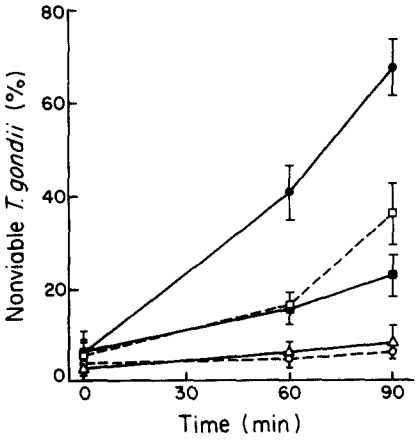

Figure 7. Toxoplasmacidal effect of CTA_2. *Toxoplasma* (2×10^7 were incubated for 90 min in the absence (○) or presence of CTA_2 (2.9×10^{-8} M, △; 2.9×10^{-7} M, ■; 2.9×10^{-6} M, □; 2.9×10^{-5} M, ●) in 2 ml PBS for 90 min at 37°C. Viability of the *T. gondii* was assessed by trypan blue staining. The data are the mean ± SE of three experiments.

Table 3. *Effect of Cyclooxygenase and Thromboxane Synthetase Inhibitors on Platelet Toxoplasmacidal Activity and TX Release*

Supplements	Nonviable *T. gondii*			TXB_2		
	%	p^1	p^2	*pg/0.1 ml*	p^1	p^2
Toxo	5.3 ± 1.6*		<0.001	4.3 ± 2.8*		<0.01
Platelets + Toxo	60.3 ± 5.3	<0.001		184.7 ± 36.6	<0.01	
+ Indo	8.9 ± 2.1		<0.001	14.0 ± 5.6		<0.02
(10^{-5} M)	(85.2)‡			(92.4)‡		
+ Indo	8.0 ± 1.2		<0.001	18.8 ± 7.6		<0.02
(10^{-6} M)	(86.7)			(89.8)		
+ Indo	22.5 ± 2.9	<0.01	<0.01	39.7 ± 13.9		<0.05
(10^{-7} M)	(62.7)			(78.5)		
+ Indo	46.0 ± 0.8	<0.001		159.0 ± 38.4	<0.02	
(10^{-8} M)	(23.7)			(13.9)		
+ Daz	10.8 ± 2.1		<0.001	16.0 ± 4.0		<0.02
(10^{-5} M)	(82.1)			(91.3)		
+ Daz	12.1 ± 2.5		<0.002	22.7 ± 9.0		<0.02
(10^{-6} M)	(79.9)			(87.7)		
+ Daz	44.2 ± 3.3	<0.001		112.0 ± 16.1	<0.01	
(10^{-7} M)	(26.7)			(39.4)		
Platelets (ASA)	8.5 ± 2.7		<0.001	0.3 ± 0.3		<0.01
+ Toxo	(85.9)			(99.8)		

The reaction mixtures contained 2×10^7 *T. gondii* (*Toxo*; RH strain) in the absence or presence of 2×10^8 platelets in 2 ml PBS. In some experiments, indomethacin (*Indo*) or dazmegrel (*Daz*) at the indicated concentrations were included in the reaction mixtures. Platelets were also obtained from individuals ingesting 1.3 mg/kg of acetylsalicylic acid (*ASA*) 2 h before blood collection. The supernatants were assayed in 0.1-ml aliquots for TXB_2 by RIA. The reaction mixtures were incubated for 90 min at 37°C with parasite viability assessed as described in Table 1.

* Mean ± SE of three experiments. Probability values for the difference from *T. gondii* alone (p^1) and platelets plus *T. gondii* (p^2) are shown where significant ($p < 0.05$).

‡ Percent inhibition.

Role of TX Release in Platelet-induced Toxicity. Inhibition of the cyclooxygenase pathway of arachidonic acid metabolism blocked platelet-mediated parasite killing (Table 3). Platelet-mediated toxoplasmacidal activity was inhibited in a concentration-dependent manner by indomethacin and dazmegrel with 85.2 and 82.1% inhibition, respectively, at a 10^{-5} M concentration of each inhibitor alone. A direct correlation between degree of inhibition of TX release and

Table 4. *Interaction of T. gondii with Human Platelet Fractions: Cytotoxicity and Release of TX*

Supplements	Nonviable *T. gondii*		TXB_2	
	%	*p*	*pg/0.1 ml*	*p*
Toxo	8.6 ± 0.9 (5)*		10.4 ± 2.5 (5)	
+ intact platelets	50.0 ± 12.5 (4)	<0.01	244.4 ± 122.0 (4)	<0.01
+ thrombin-stimulated platelets	39.1 ± 4.8 (3)	<0.001	197.3 ± 68.1 (3)	<0.01
+ freeze-thawed platelets:				
entire contents	30.4 ± 2.7 (3)	<0.001	130.7 ± 11.6 (3)	<0.001
100,000-*g* pellet	44.5 ± 13.2 (3)	<0.02	214.3 ± 65.6 (3)	<0.01
100,000-*g* supernatant	10.1 ± 0.5 (3)		19.3 ± 4.1 (3)	

The reaction mixture was as described for Table 1 except the following subcellular fractions representing material obtained from 2×10^8 human platelets (prepared as described in Materials and Methods) were assayed for cytotoxicity against *T. gondii* (*Toxo*; 2×10^7) in a final volume of 2 ml for 90 min at 37°C as follows: thrombin (5 U/ml)-stimulated platelets or the following fractions of platelets disrupted by freezing and thawing: entire contents; 100,000 *g* pellet (microsomes); and 100,000-*g* supernatant (microsome depleted). Control *Toxoplasma* were incubated in buffer alone for 90 min at 37°C. Viability of organisms was assessed by trypan blue staining, and TXB_2 was assayed by RIA.

* Mean ± SE of experiments (*n*). Probability (*p*) values for the difference from *T. gondii* alone are shown where significant ($p < 0.05$).

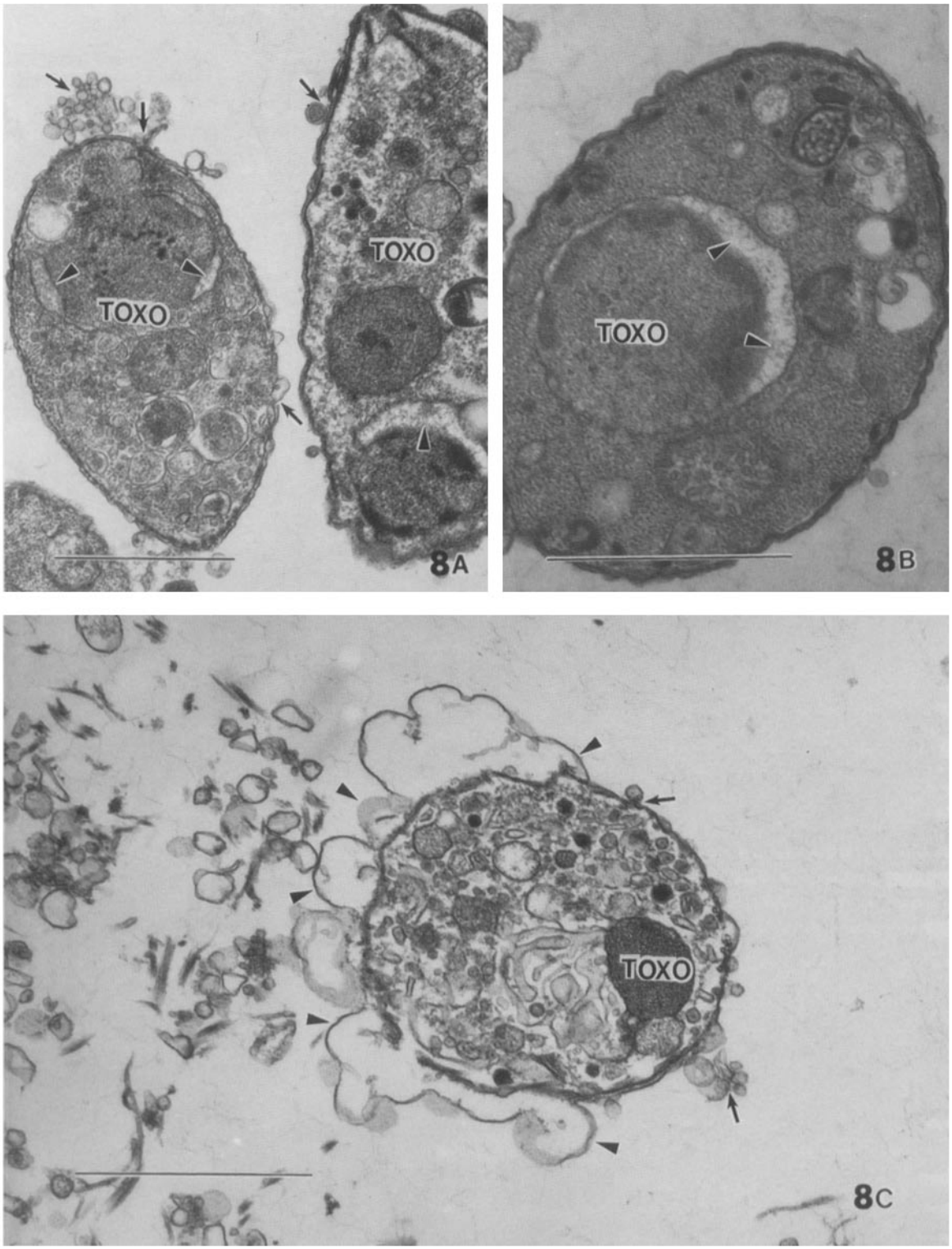

Figure 8. Effect of CTA_2 and platelet microsomes on *T. gondii* morphology. *Toxoplasma* (*TOXO*) were incubated in buffer for 90 min at 37°C with either (*A* and *B*) 2.9×10^{-5} M CTA_2 as described in Fig. 7 or (*C*) the 100,000 *g* pellet (microsomes) obtained from 2×10^8 platelets that had been disrupted by freezing and thawing as described in Table 4. As seen in *A*, CTA_2 caused marked vesiculization of the surface membrane (*arrows*) and dilation of the nuclear envelope (*arrowheads*) of the *Toxoplasma*. Lysis of *Toxoplasma* by the platelet microsome-containing particulate fraction is observed in *C*. Extensive swelling (*arrowheads*) and disruption of surface membranes with loss of intracytoplasmic contents are noted. Surface membrane vesiculization (*arrows*) is also evident. Bars, 1 μm. (*A*) ×30,000; (*B*) ×42,000; (*C*) ×40,000.

cytotoxicity induced by either indomethacin or dazmegrel was observed (Table 3). Further, platelets obtained from individuals who had ingested acetylsalicylic acid before blood collection failed to either exert a significant cytotoxic effect or release TXB_2 after interaction with *Toxoplasma* (Table 3).

Since TXA_2 is unstable with a short half-life of ~30 s at pH 7.4 and 37°C (47), the stable TX analogue CTA_2 (48) and a platelet microsomal TXA_2-generating system were examined for toxoplasmacidal activity. Incubation of *Toxoplasma* with 2.9×10^{-7} M CTA_2 for 90 min resulted in killing of 22.7% of the organisms; cytotoxicity increased to 67.4% at 2.9×10^{-5} M CTA_2 (Fig. 7). Ultrastructural studies (Fig. 8, *A* and *B*) showed that CTA_2 (2.9×10^{-5} M) induced vesicle formation in the surface membranes of the *Toxoplasma* and dilation of the perinuclear membranes of the organisms.

The entire contents of platelets disrupted either by treatment with 5 U/ml thrombin for 15 min or by three cycles of freezing and thawing over a 90-min period which completely releases the platelet cytoplasmic granule contents as previously shown (14) caused a significant reduction in the viability of the *Toxoplasma* and the respective release of 197.3 and 130.7 pg of TXB_2 per 0.1-ml sample (Table 4). The platelets that had been disrupted by freezing and thawing were centrifuged at 5,000 *g* for 15 min. Additional centrifugation of the supernatant at 100,000 *g* for 60 min produced a microsome-containing particulate fraction and a microsome-depleted supernatant as previously demonstrated (31). TX synthetase, which is located in platelet microsomes (31, 32), converts the unstable cyclic endoperoxides PGG_2 and PGH_2 to TXA_2 (31, 47). The presence of TXA_2 synthetase in the microsome preparations was confirmed by the recovery of 398.0 ± 166.3 pg TXB_2 per 0.1-ml sample of the 100,000-*g* pellet after incubation with 10^{-5} M PGH_2 for 90 min at 37°C (n = 3). The microsome-containing 100,000-*g* pellet induced a significant increase in nonviability of the organisms. Reaction mixtures containing the platelet 100,000-*g* particulate fraction and *T. gondii* released 20.6-fold greater amounts of TXB_2 than did *T. gondii* alone (Table 4). In contrast, the 100,000-*g* supernatant was ineffective in killing the *Toxoplasma* and did not induce a significant increase in TXB_2 release compared with reaction mixtures with *T. gondii* alone (Table 4). The toxoplasmacidal activity of the platelet microsomal fractions was confirmed by transmission electron microscopy. *T. gondii*, after incubation with platelet microsomes (Fig. 8 *C*), exhibited such cytotoxic changes as swelling of their surface membranes and disruption of their intracellular organelles.

Discussion

These studies demonstrate that human platelets exert potent cytotoxic activity against tachyzoites (i.e., the proliferating form) of the pathogenic protozoan, *T. gondii*. Platelet toxoplasmacidal activity, like platelet tumoricidal activity (21), occurs in the absence of added antibody. This is in contrast to platelet-mediated ADCC against erythrocytes (13, 14) and infective forms of *S. mansoni* (16) and *B. malayi* (19) which require either IgG or IgE antibody for cytotoxicity. Further, heat-inactivated sera containing *Toxoplasma*-specific IgG antibodies in the absence or presence of *Toxoplasma*-specific IgM antibodies had no effect on platelet-mediated toxoplasmacidal activity. Platelet-mediated killing of *Toxoplasma* occurs at platelet/*T. gondii* ratios as low as 1:3 with toxicity increasing with higher ratios of platelets to organisms over a 90-min period. Prior studies assayed platelet-mediated tumor cell cytotoxicity after a 48-h incubation period (21). Human platelet–induced killing of *T. gondii* appears to require close contact between effector and target cells since separation of platelets from *Toxoplasma* by a membrane filter abrogated cytotoxicity. Ultrastructural studies demonstrated initial adherence of platelet pseudopodal projections to the surface of the parasites which was followed by swelling of the outer parasite membrane and intracellular cytolysis of the *T. gondii*. *Toxoplasma* after their interaction with platelets in vitro were unable to infect mice in vivo.

Destruction of *T. gondii* by human platelets was not mediated by the release of toxic oxygen species by the platelets since O_2^- generation by platelets after interaction with *Toxoplasma* was not observed and CGD platelets were cytotoxic to the organisms. In addition, platelet granule contents were not implicated in the cytotoxic process since the microsome-depleted supernatant fractions containing granule material obtained from platelets disrupted by freezing and thawing lacked toxoplasmacidal activity. Slezak et al. (14) were similarly unable to demonstrate a role for either platelet-derived oxygen radicals or granule components in platelet-mediated ADCC against sheep erythrocytes. Our data suggest that TX generation may be important in the mediation of platelet killing of *Toxoplasma*. Evidence in support of this role for TX is as follows.

There was a 3.9-fold increase in TXB_2 release by platelets incubated with *T. gondii* (1:1 ratio) compared with platelets incubated in buffer alone. Inhibition of platelet cyclooxygenase either in vitro by indomethacin or in vivo by acetylsalicylic acid inhibited platelet-mediated parasite killing and release of TXB_2. Further, the selective TX synthetase inhibitor dazmegrel abrogated in vitro platelet-mediated killing of *Toxoplasma*. The selectivity of the inhibitory effect of dazmegrel on TX synthetase vs. cyclooxygenase enzyme activity was demonstrated by the inhibitory effect of dazmegrel on TXB_2 release in contrast to its stimulatory effect on PGE_2 release by the platelet–*T. gondii* reaction mixtures. Although *T. gondii* readily incorporate exogenously added arachidonic acid, the *Toxoplasma* do not convert arachidonate into lipoxygenase products (26). Generation of PGE_2 in the platelet–*T. gondii* preparations was presumably from the organisms since *T. gondii* incubated in buffer alone for 90 min released 73.4 pg PGE_2 per 0.1-ml sample containing 10^6 *Toxoplasma*. Whereas other parasites such as *Taenia taeniaeformis* (49, 50) and *S. mansoni* (51), as well as gram-negative and gram-positive bacteria (52), release PGE_2 and other eicosanoids, formation of cyclooxygenase arachidonate products by *T. gondii* has not been described previously to our knowledge.

Additional evidence for TXA_2 in the mediation of platelet-induced cytotoxicity against *T. gondii* was provided

by studies examining the cytotoxic effect of the TX analogue CTA_2 and microsome-containing fractions of disrupted platelets. Although CTA_2 lacks the platelet aggregatory activity of TXA_2, this stable TX analogue does exhibit the potent vasoconstrictor activity of biologically generated TXA_2 (53, 54). Prior studies in cats have demonstrated that CTA_2 infusion damages myocardial cell membranes with release of myocardial creatine kinase and lysosomal hydrolase activities (54). We found that CTA_2 and platelet microsomes capable of releasing TX produced extensive surface and perinuclear membrane damage in the *Toxoplasma* and induced significant nonviability of the organisms. Since *T. gondii* form PGE_2, it is possible that the organisms provide the cyclic endoperoxide precursor (PGG_2/PGH_2) required by TXA_2 synthetase present in the platelet microsomes to generate TX.

CTA_2-induced *Toxoplasma* cytotoxicity was observed beginning at a concentration of 2.9×10^{-7} M which is comparable to the 2×10^{-7} M CTA_2 concentration that stimulates the release of lysosomal hydrolases from large granule fractions of liver homogenate (53). In the platelet–*T. gondii* (1:1 ratio) reaction mixtures, generation of 4×10^{-9} M TX (Fig. 6) was associated with significant parasite cytotoxicity (Table 1). The greater reactivity of TX in comparison to CTA_2 in induction of *Toxoplasma* cytotoxicity may result from a unique structural feature of TXA_2. TXA_2 has an acetal carbon atom that binds two oxygen molecules in an extremely strained bicyclic structure that is susceptible to attack by nucleophiles (47), whereas CTA_2 is lacking in this structure (53).

TXA_2 release by platelets and other inflammatory cells may have additional important effects on the immune response to *Toxoplasma* besides a direct cytolytic effect. As reported by Tripp et al., (55), TXA_2 synthesis is preferentially conserved in *Listeria monocytogenes*–infected murine peritoneal macrophages. In their studies, 100% of *Listeria*-infected mice died when treated with indomethacin, in contrast to no deaths of animals in the absence of cyclooxygenase blockade (56). Further, indomethacin-induced dissemination of *Listeria* was reversed by administration of a stable TXA_2 analogue suggesting that TXA_2-induced vasoconstriction may help localize the organisms to the initial site of infection (56).

Our studies demonstrate a novel cytotoxic role for human platelets. Platelet adherence to the surface of *T. gondii* may occur during parasitemia or at inflammatory sites of toxoplasmal infection. We are currently investigating what factors promote this contact. Such interaction may result in platelet aggregation and release of TXA_2 and other products which induce cytotoxic damage to the organisms, thus contributing to the host defense against this pathogen.

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