Enzymatic treatment transforms trypomastigotes of *Trypanosoma cruzi* into activators of alternative complement pathway and potentiates their uptake by macrophages

(complement system/serine proteases/sialidase/parasites/protozoa)

THEREZA L. KIPNIS*†, JOHN R. DAVID*†, CHESTER A. ALPER‡§, ALAN SHER¶, AND W. DIAS DA SILVA‡§

Departments of *Medicine and ¶Pathology, Harvard Medical School; †Department of Immunology and Rheumatology, the Brigham and Women's Hospital; ‡Department of Pediatrics, Harvard Medical School; and §Center for Blood Research, Boston, Massachusetts 02115

ABSTRACT   In the absence of bound antibody, trypomastigote bloodstream forms of *Trypanosoma cruzi* fail to activate the alternative complement pathway. We now demonstrate that treatment with trypsin and, to a lesser extent, with sialidase converts these protozoa into activators of the pathway, as judged by their lysis in normal sera or sera genetically deficient in fourth or second component of complement (C4 or C2) and their Mg^{2+}-dependent consumption of C3 as measured by crossed immunoelectrophoresis. In addition, after pretreatment with enzyme and incubation in C5-deficient serum, trypomastigotes were shown to possess both C3 and properdin factor B (B) on their surface as judged by immunofluorescence. Requirement for the late components C5–C9 was suggested by the failure of C5-deficient sera to lyse trypsin-treated parasites. The inability to activate the alternative complement pathway was regained by these organisms after incubation *in vitro*. This restoration of insusceptibility was inhibited when puromycin was included in the culture medium. Treatment of the trypomastigotes with trypsin also potentiated their uptake by mouse peritoneal macrophages without apparent interference with their capacity to differentiate and multiply inside the cell. These findings suggest that untreated trypomastigotes normally escape recognition by the alternative pathway *in vivo* because of the presence on their surface of trypsin- and sialidase-sensitive regulatory molecules, the expression of which is dependent on protein synthesis.

Infection with *Trypanosoma cruzi* is initiated by trypomastigotes which differentiate from epimastigotes in the gut of the insect vector (1). Epimastigotes are lysed by normal serum (2) through the activation of the alternative complement pathway (ACP) (3). In contrast, trypomastigotes obtained from cultures (3) or from the blood of irradiated mice (4) are resistant to ACP lysis in the absence of antibody. Therefore, the differentiation of epimastigotes into trypomastigotes is apparently accompanied by a modification of the parasite surface that renders it resistant to ACP lysis.

It has been demonstrated that cells activating ACP have membrane components that protect the activated form of third component of complement (C3b) deposited on their surface from inactivation by C3b inactivator (C3bINA) and β1H and that stabilize C3 convertase (C3bBb) from dissociation by β1H (5). Removal of membrane sialic acid residues from sheep erythrocytes by sialidase enables these cells to activate ACP (6, 7) and makes them susceptible to phagocytosis by human monocytes (8). In addition, coupling with heparin glycosaminoglycan impairs the ability of zymosan to activate the ACP (9).

We have studied whether regulatory mechanisms similar to those described above explain the inability of *T. cruzi* trypomastigotes to activate the ACP. Our results demonstrate that

trypomastigotes treated with trypsin or sialidase become able to activate ACP and are lysed. The trypsin-treated trypomastigotes recover resistance to ACP lysis within 6 hr. This recovery is inhibited, however, when puromycin is added to the culture medium. Treating the trypomastigotes with trypsin also results in an increase in their uptake by macrophages but does not affect their intracellular survival and multiplication.

MATERIALS AND METHODS

Parasites. Bloodstream trypomastigotes were obtained from γ-irradiated [650 R (0.17 coulomb/kg)] A/J mice (The Jackson Laboratory, Bar Harbor, ME) infected with the CL strain of *T. cruzi*. Trypomastigotes were isolated from heparinized blood by differential centrifugation (10) and suspended at $1-2 \times 10^6$ organisms per ml in minimal essential medium (GIBCO) containing 100 units of penicillin, 100 μg of streptomycin, and 5 units of heparin per ml. Where indicated, the medium was supplemented with 10% fetal calf serum.

Complement (C) Reagents. Normal human serum was obtained from healthy donors living in areas not endemic for Chagas disease. Normal guinea pig serum and normal rat serum were obtained from noninfected laboratory animals. Sera were obtained from patients genetically deficient in C2, C3, and C5. Guinea pig serum genetically deficient in C4 and purified human C4 and C5 were provided by H. Colten (Children's Hospital Medical Center, Boston, MA). Human C2, C3, and properdin factor B (B) were purified by described methods (11–13).

Antisera. Monospecific goat antisera against human C3 and fluorescein conjugates of rabbit anti-human C4, B, C3, and β1H were from Atlantic Antibodies (Scarborough, ME).

Enzymes, Enzyme Inhibitors, and Reagents. Trypsin type III (twice crystallized, from bovine pancreas), elastase, α-chymotrypsin type VII (TLCK treated), soybean trypsin inhibitor (SBTI), N^α-*p*-tosyl-L-lysyl chloromethyl ketone (TLCK), α_1-trypsin inhibitor, and puromycin were obtained from Sigma. Purified thrombin (2.1×10^4 units/mg) was a gift from D. H. Bing (Center for Blood Research, Boston, MA); *Clostridium perfringens* sialidase (acylneuraminyl hydrolase, EC 3.2.1.18; 16 units/mg) was a gift from D. T. Fearon (Brigham and Women's Hospital, Boston, MA). The proteolytic enzymes were dissolved in minimal essential medium at 1 mg/ml; sialidase was dissolved in the same medium at pH 6.5 at 5 units/ml. EDTA and ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate were obtained from Fisher.

Abbreviations: ACP, alternative complement pathway; C, complement system; C3, third component of C; C3b, activated form of C3; C3bINA, C3b inactivator; B, properdin factor B; C3bBb, C3 convertase; TLCK, N^α-tosyllysine chloromethyl ketone; SBTI, soybean trypsin inhibitor.

Immunologic Techniques. C3 cleavage was measured by crossed immunoelectrophoresis in 0.8% agarose/barbital-buffered saline (pH 8.6; ionic strength, 0.056) and containing 1 mM EDTA (VBS-EDTA) (14). Deposition of C components on trypanosome cell surfaces was assayed by immunofluorescence. In the assay, 100 μl of trypomastigote suspension was incubated with an equal volume of C5-deficient human serum for 30 min at 37°C. The parasites were then washed twice with minimal essential medium and reincubated with 100 μl of a 1:40 dilution of fluorescein conjugated rabbit-anti human C4, B, C3, C5, and β1H for 30 min at room temperature. After an additional series of washings, the parasites were examined at $\times$1000 magnification with a Leitz fluorescence microscope.

Treatment of Bloodstream Trypomastigotes with Enzymes. To 900 μl of trypanosome suspension in minimal essential medium was added 100 μl of enzyme solutions at pH 7.6 for the proteolytic enzymes and at pH 6.5 for sialidase. The mixtures were incubated for 30 min at 37°C; then 100 μl of SBTI (1 mg/ml) or minimal essential medium/fetal calf serum was added, and the mixtures were reincubated for an additional 10 min at 37°C. After washing with minimal essential medium/fetal calf serum, the enzyme-treated trypanosomes were adjusted to 1–2 $\times$ 10^6/ml.

Lytic Assay. To measure lysis by C, 100 μl of control or enzyme-treated parasites was mixed with 100 μl of normal serum and incubated for 30 min at 37°C. Aliquots were examined in a hemocytometer before and after the incubation with C. The number of viable trypomastigotes was expressed as the mean $\pm$ SD of replicated tubes. C3 conversion was measured in centrifuged supernatant medium from the cultures.

Mouse Peritonal Macrophage Cultures. Peritoneal macrophages were induced in mice by intraperitoneal injections of 1.5 ml of sterile 10% Bacto Proteose peptone no. 3 (Difco). These cells were used to form monolayers on 15-mm round coverslips in 24-well tissue culture plates (Costar, Cambridge, MA). After 24 hr of incubation the monolayers were overlaid with 300 μl of a trypanosome suspension, incubated for 1 hr at 37°C in an atmosphere of 5% CO_2/95% air, and then rinsed twice in minimal essential medium to remove the extracellular parasites. After washing, the macrophages were overlaid with fresh minimal essential medium/fetal calf serum and reincubated for an additional 48 hr. To evaluate intracellular infection, the macrophage cultures were washed twice with phosphate-buffered saline, fixed in methanol, and stained with Giemsa solution (Fisher). After drying, the coverslips were mounted on microscope slides. Macrophages in 10 random $\times$400 microscopic fields were examined, and the percentage of infected cells and the mean number of intracellular *T. cruzi* per 100 macrophages were calculated.

RESULTS

Lysis by Normal Serum. Trypomastigotes treated with trypsin or sialidase were lysed by complement in normal human serum, but trypanosomes untreated or treated with thrombin, elastase, or α-chymotrypsin were resistant to lysis (Table 1). In other experiments, trypomastigotes treated with trypsin (but not untreated trypomastigotes) were also lysed by normal guinea pig serum and normal rat serum. Because trypsin treatment was the most active in promoting C-mediated lysis of trypanosomes, this enzyme was used in subsequent experiments.

Trypsin cleaves C3 into two major products resembling C3b and C3a (15). Because binding of trypsin to the trypanosome surface might activate the membrane attack complex C5b–C9, we investigated C lysis of trypsin-treated bloodstream forms in the presence of the inhibitors SBTI, TLCK, and α_1-trypsin inhibitor. When used at nontoxic concentrations (42.5 μg/ml)

Table 1. Susceptibility of enzyme-treated trypomastigotes of *T. cruzi* to lysis by complement

Enzyme	Culture medium*	Trypanosomes, no. $\times$ 10^{-4}/ml†		% lysis
		Before incubation	After incubation	
—	MEM/FCS	36.6 $\pm$ 4.9	36.6 $\pm$ 2.0	0.0
Sialidase	MEM/FCS	23.5 $\pm$ 0.7	21.5 $\pm$ 0.7	8.6
	NHS	23.7 $\pm$ 3.4	9.8 $\pm$ 1.8	68.0
Trypsin	MEM/FCS	34.5 $\pm$ 0.7	36.0 $\pm$ 1.4	0.0
	NHS	33.0 $\pm$ 1.4	0	100.0
Thrombin	MEM/FCS	19.5 $\pm$ 0.7	26.0 $\pm$ 1.4	0.0
	NHS	33.5 $\pm$ 0.7	27.5 $\pm$ 0.7	18.0
Elastase	MEM/FCS	32.0 $\pm$ 2.8	35.5 $\pm$ 0.7	0.0
	NHS	34.0 $\pm$ 1.4	36.5 $\pm$ 3.5	0.0
α-Chymotrypsin	MEM/FCS	45.0 $\pm$ 0.7	51.5 $\pm$ 3.5	0.0
	NHS	45.5 $\pm$ 3.5	54.0 $\pm$ 5.6	0.0

* MEM/FCS, minimal essential medium/fetal calf serum; NHS, normal human serum.
† Each value represents the mean $\pm$ SD of triplicate samples.

with 1.6–1.9 $\times$ 10^4 trypanosomes, these agents did not alter the complete lysis of the trypsin-treated trypomastigotes by C.

Regeneration of Resistance to C Lysis After Trypsin Treatment. A suspension of 1 $\times$ 10^6 trypsin-treated trypanosomes per ml was incubated for 6 hr at 37°C in minimal essential medium/fetal calf serum in either the presence or absence of puromycin at 10 μg/ml. Aliquots (100 μl) were withdrawn after 0, 3, 5, and 6 hr of incubation and added to 100 μl of normal
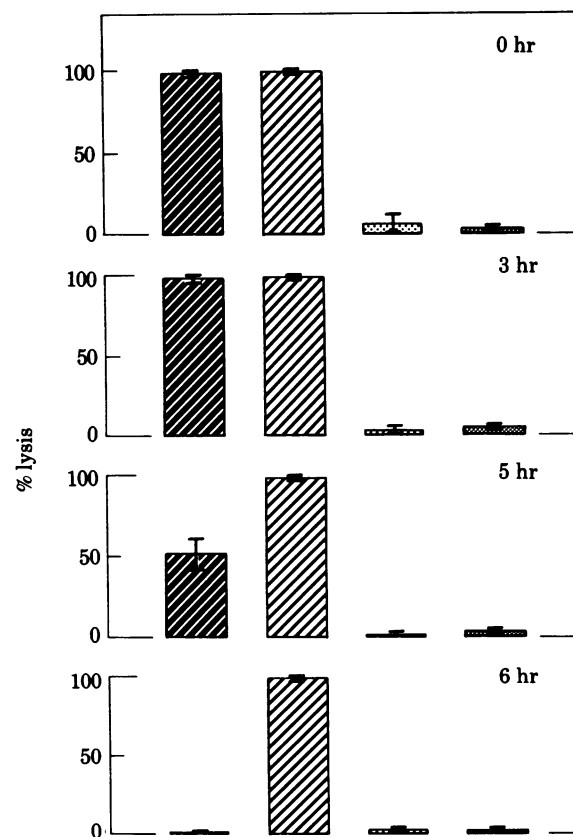

FIG. 1. Trypomastigotes were treated with trypsin and then incubated in either minimal essential medium/fetal calf serum (▨) or minimal essential medium/fetal calf serum + puromycin (▩). Control cultures consisted of untrypsinized parasites incubated in the same media (▒ or ▦, respectively). Each value is the mean ($\pm$ SD) % lysis after incubation for 30 min in C; n = four determinations.

Table 2. Requirement of C components for lysis of bloodstream trypomastigotes treated with trypsin

Serum*	Reconstituted with	Trypanosomes, no. × 10⁻⁴/ml†		% lysis
		Before incubation	After incubation	
MEM/FCS	None	179.0 ± 6.3	184.0 ± 8.4	0.0
NHS	None	156.0 ± 1.0	0	100.0
NHS, 56°C, 1 hr	None	192.0 ± 11.3	206.0 ± 14.1	0.0
NHS, 52°C, 1 hr	None	187.0 ± 4.2	204.0 ± 5.6	0.0
NHS, 52°C, 1 hr	B	100.0 ± 2.8	38.6 ± 2.0	62.0
NHS, 52°C, 1 hr	C2	75.3 ± 16.9	72.0 ± 15.1	5.0
NHS, 52°C, 1 hr	B + C2	75.5 ± 13.4	22.6 ± 3.7	70.0
C2-deficient human serum	None	52.5 ± 3.5	24.5 ± 0.7	52.0
C4-deficient guinea pig serum	None	34.6 ± 13.6	8.3 ± 1.5	76.0
C4-deficient guinea pig serum	C4	36.0 ± 7.0	13.6 ± 1.5	63.0
C3-deficient human serum	None	52.6 ± 1.5	53.3 ± 0.5	0.0
C3-deficient human serum	C3	53.6 ± 3.0	3.6 ± 0.5	94.5
C5-deficient human serum	None	56.3 ± 3.2	60.0 ± 1.4	0.0
C5-deficient human serum	C5	59.0 ± 1.0	44.3 ± 6.1	20.8
NHS/5 mM EGTA/2 mM Mg²⁺	None	123.0 ± 11.2	0	100.0
NHS/6 mM EDTA	None	171.6 ± 11.6	190.0 ± 9.8	0.0

* See Table 1 for abbreviations. EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
† Reconstitution was by adding homologous C components to the C-depleted serum at the following final concentrations: C4, 100 μg/ml; B, 10 μg/ml; C2, 5 μg/ml; C3, 200 μg/ml; C5, 10 μg/ml. Each value represents the mean ± SD of triplicate samples.

human serum to determine susceptibility to ACP lysis. A control suspension of trypomastigotes was processed in the same way except that the parasites were not trypsinized. Trypsin-treated trypanosomes recovered resistance to ACP lysis after 6 hr of incubation. This recovery was blocked by puromycin (Fig. 1). At the concentration used, puromycin was not toxic for the parasites and did not promote ACP lysis of the nontrypsinized trypomastigotes.

Requirement of C Components for Lysis of Trypsin-Treated Trypomastigotes. Lysis of the trypanosomes occurred in sera depleted or genetically deficient in the classical C components C2 and C4 (Table 2). However, depletion of B by heating the serum at 52°C blocked the lysis of trypanosomes; the trypanocidal activity was restored by the addition of purified B but not C2. As predicted, C3-deficient human serum also failed to lyse trypanosomes, and the activity was restored by the addition of purified human C3. Crossed immunoelectrophoresis patterns of the supernatant sera incubated with trypanosomes showed that C3 was converted by the trypsin-treated organisms. The results of the experiments with chelators showed that lysis of trypanosomes by normal human serum requires Mg²⁺ but not Ca²⁺, thus providing additional evidence for the ACP as the C pathway involved in lysis of the trypanosomes. C5-deficient human serum also failed to lyse trypsin-treated trypomastigotes, but the addition of purified human C5 partially restored the trypanocidal capacity, thus indicating that at least some of the later-reacting components are necessary for trypanosome lysis.

Finally, in order to determine which C components were actually on the cell membrane, trypsin-treated bloodstream forms were incubated with C5-deficient human serum or with heat-inactivated C5-deficient human serum for 30 min at 37°C. After washing, the parasites were treated with fluorescein-conjugated specific antiserum to C4, B, C3, C5, or β1H. Trypanosomes incubated with the heat-inactivated C5-deficient human serum did not fluoresce. However, more than 80% of trypomastigotes incubated with unheated serum fluoresced with anti-C4, -B, and -C3. C5 and β1H were not detected on the parasites.

Interaction of Trypsin-Treated Trypomastigotes and Mac-

rophages. Trypsin treatment of trypomastigotes of the CL strain more than doubled their uptake by monolayers of mouse peritoneal macrophages when measured after 1 hr of contact (Fig. 2). This enhanced uptake was reflected both in the percentage of macrophages infected and in the number of intracellular *T.*
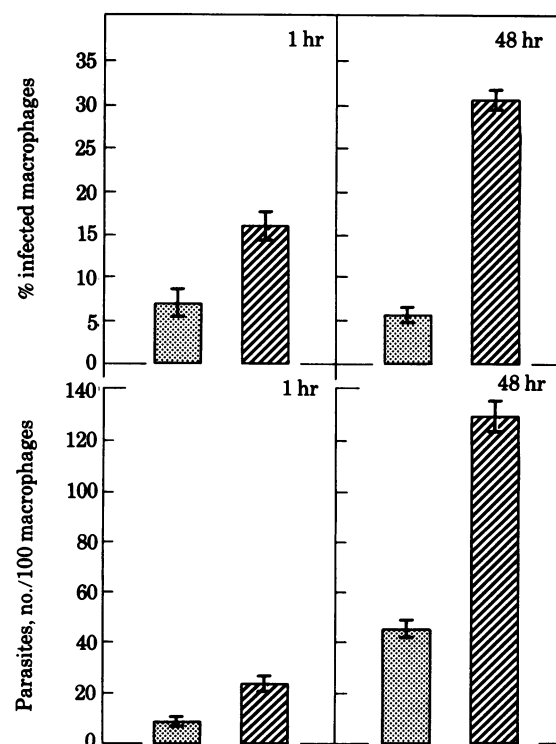

FIG. 2. Effect of trypsin treatment on the uptake of trypomastigotes by macrophages. Trypomastigotes either untreated (▒) or pretreated with trypsin (▨) were incubated at 37°C with mouse peritoneal macrophages in minimal essential medium/fetal calf serum for 1 hr, washed, and then incubated for an additional 48 hr in the same medium. The values shown represent the mean ± SD of determinations on four replicate cultures in each of which at least 500 cells were examined.

cruzi per 100 macrophages. (In data not shown, extending the period of contact to 4 hr further accentuated the differential uptake of trypsin-treated parasites compared to nontreated controls.) The eventual intracellular fate of the trypsin-treated trypanosomes was evaluated after 48 hr of incubation. Treated bloodstream forms multiplied and differentiated as nontreated trypomastigotes. The greater number of intracellular parasites compared to nontreated trypanosomes reflected the greater uptake of the former.

DISCUSSION

Two mechanisms of natural immunity against *T. cruzi* which could operate in the unsensitized host are the cytolytic effect of ACP (16) and the antibody-independent recognition by phagocytic cells (8). One critical event in the activation of ACP is the circumvention of the regulation of membrane-bound C3b by proteins β1H and C3bINA which prevent the formation of stabilized C3 convertase. It appears that the surface of ACP-activating particles provides greater protection of C3b-bound molecules against inactivation by these regulatory proteins than nonactivating cells (5). Experimental manipulation of the membrane surface of nonactivators of ACP has enabled the beginning of the biochemical characterization of the environment containing C3b-bound molecules. For example, with sheep erythrocytes, partial enzymatic removal of membrane sialic acid residues or their chemical conversion to heptulosonic acid derivatives (6, 7) enables these cells to activate the ACP. Conversely, zymosan, which is an activator of ACP, can be made resistant by coupling heparin glycosaminoglycan onto the particle surface (9). This suggests that surface components, probably glycoproteins, with a specific structure and conformation regulate the interaction of bound C3b with β1H.

Similar mechanisms may control the activation of C by *T. cruzi*. Thus, sialic acid residues (17) and a lipopeptidophosphoglycan have been identified on culture forms of *T. cruzi* (18). The activation of the ACP by enzymatically treated trypomastigotes observed in our experiments may be due therefore to the cleavage, by trypsin or sialidase, of surface components bearing either of these moieties.

That lysis was indeed dependent on the activation of the ACP was suggested by the experiments using sera genetically deficient in C2, C4, and C3 and sera treated to inactivate B or C2 or to remove Ca^{2+} (Table 2). In addition, the C3b cleavage product of ACP activation was demonstrated in the fluid phase after incubation of enzyme-treated trypomastigotes with normal serum. Finally, ACP activation was also indicated by our demonstration of membrane fluorescence with antisera to B and C3 on the surface of trypsin-treated parasites preincubated in C5-deficient human serum. Although activation of the classical pathway is not necessary for the lysis of trypsin-treated trypomastigotes, we did observe C4 on the parasite surface. C4 deposition could result from C1 activation by nonspecific binding of C1q on the parasite surface, a mechanism that has been described for the lysis of retroviruses by C (19). C4 deposition is unlikely to be due to IgG or IgM because no immunoglobulin was seen on the trypanosomes by immunofluorescence.

The trypsin and sialidase sensitivities of the surface membrane component protecting the trypomastigotes from ACP activation suggest that it could be a glycoprotein. That the component is a protein was also suggested by puromycin inhibition of recovery of resistance to ACP-mediated lysis (Fig. 1).

Recent studies have demonstrated a trypsin-sensitive receptor on human monocytes that is capable of recognizing surfaces that activate the ACP (8). Trypomastigotes, particularly from strains containing predominantly stout forms such as the CL strain used in this study, are taken up poorly by macrophages (20, 21). The enhanced uptake of trypsin-treated trypomastigotes by macrophages suggests similarities between the components recognized by both humoral (i.e., ACP) and cellular (i.e., macrophage) defense mechanisms. In this regard, it should be emphasized that, although treatment of trypomastigotes with trypsin increased their uptake by macrophages, the intracellular survival of the parasites was not impaired. They were found to multiply and differentiate as readily as nontreated trypomastigotes (Fig. 2).

In summary, our findings provide evidence for the existence of surface molecules modulating the activation of the ACP during the life cycle of *T. cruzi*. In the process of seeking a new host cell, *T. cruzi* is extracellular and is thus exposed to C lysis activated by the ACP. The infective trypomastigote stage, but not the noninfective epimastigote stage, has developed membrane components that do not activate the ACP. In the immune host, however, antibody-dependent lysis of trypomastigotes occurs by ACP activation of C (4, 22). It is possible that antibodies function in a fashion similar to that of trypsin and sialidase by modifying membrane components that prevent the organisms from activating the ACP.

1. Dias, E. (1934) *Mem. Inst. Oswaldo Cruz* 28, 1–110.
2. Muniz, J. & Borriello, A. (1945) *Rev. Bras. Biol.* 5, 563–576.
3. Nogueira, N., Bianco, C. & Cohn, Z. (1975) *J. Exp. Med.* 142, 224–229.
4. Krettli, A. U., Weisz-Carrington, P. & Nussenzweig, R. S. (1979) *Clin. Exp. Immunol.* 37, 416–423.
5. Fearon, D. T. & Austen, K. F. (1977) *Proc. Natl. Acad. Sci. USA* 74, 1683–1687.
6. Fearon, D. T. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1971–1975.
7. Pangburn, M. K. & Muller-Eberhard, H. J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2416–2420.
8. Czop, J. K., Fearon, D. T. & Austen, K. F. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3831–3835.
9. Kazatchkine, M. D., Fearon, D. T., Silbert, J. E. & Austen, K. F. (1979) *J. Exp. Med.* 150, 1202–1215.
10. Okabe, K., Kipnis, T. L., Calich, V. L. G. & Dias da Silva, W. (1980) *Clin. Immunol. Immunopathol.* 16, 1062–1071.
11. Polley, M. J. & Muller-Eberhard, H. J. (1968) *J. Exp. Med.* 128, 533–551.
12. Tack, B. F. & Prahl, J. W. (1976) *Biochemistry* 15, 4513–4521.
13. Gotze, O. & Muller-Eberhard, H. J. (1971) *J. Exp. Med.* 134, 90s–108s.
14. Laurell, C. B. (1965) *Anal. Biochem.* 10, 358–361.
15. Nilsson, U. A., Mandle, R. J., Jr.. & McConnell-Mapes, J. A. (1975) *J. Immunol.* 114, 815–822.
16. Wardlaw, A. C. & Pillemer, L. (1956) *Ann. N.Y. Acad. Sci.* 66, 244–246.
17. Alves, M. J. M. & Colli, W. (1975) *FEBS Lett.* 52, 188–190.
18. De Lederkremer, R. M., Tanaka, C. T., Alves, M. J. M. & Colli, W. (1977) *Eur. J. Biochem.* 74, 263–267.
19. Cooper, N. R., Jensen, F. C., Welsh, R. M., Jr. & Oldstone, M. B. A. (1976) *J. Exp. Med.* 144, 970–984.
20. Kipnis, T. L., Calich, V. L. G. & Dias da Silva, W. (1979) *Parasitology* 75, 89–98.
21. Alcantara, A. & Brener, Z. (1978) *Acta Tropica* 35, 209–219.
22. Budzko, D. B., Pizzimenti, M. C. & Kierszenbaum, F. (1975) *Infect. Immun.* 11, 86–91.