Two Forms of Autosomal Chronic Granulomatous Disease Lack Distinct Neutrophil Cytosol Factors

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Chronic granulomatous diseases of childhood (CGD) are a group of disorders of phagocytic cell superoxide ($O_2^{\cdot-}$) production (respiratory burst). Anion exchange chromatography separated from normal neutrophil cytosol a 47-kilodalton neutrophil cytosol factor, NCF-1, that restored activity to defective neutrophil cytosol from most patients with autosomally inherited CGD in a cell-free $O_2^{\cdot-}$-generating system. A 65-kilodalton factor, NCF-2, restored activity to defective neutrophil cytosol from one patient with autosomal CGD. NCF-1, NCF-2, and a third cytosol fraction, NCF-3, were inactive alone or in pairs, but together replaced unfractionated cytosol in cell-free $O_2^{\cdot-}$ generation. Neutrophils deficient in NCF-1, but not NCF-2, did not phosphorylate the 47-kilodalton protein. It is proposed that NCF-1, NCF-2, and NCF-3 are essential for generation of $O_2^{\cdot-}$ by phagocytic cells and that genetic abnormalities of these cytosol components can result in the CGD phenotype.

CHRONIC GRANULOMATOUS DISEASES of childhood (CGD) are a genetically heterogeneous group of disorders of phagocytic cell superoxide ($O_2^{\cdot-}$) production that result in defective microbicidal activity, recurrent infections, and excessive granuloma formation. CGD occurs with X-linked inheritance (66%) or with autosomal inheritance (33%) (*1*).

Polymorphonuclear neutrophilic leukocytes (PMNs) from most X-linked CGD patients lack a membrane-associated cytochrome b_{558}, required for production of $O_2^{\cdot-}$ (*1, 2*). The gene responsible for this X-linked form of CGD codes for the large subunit of cytochrome b_{558} (*3*). Most patients with autosomally inherited CGD generally have PMNs that contain cytochrome b_{558} (*1, 4*).

The nature of the defect in the majority of cases of autosomal recessive, cytochrome b_{558}–positive CGD (AR^+ CGD) has remained unclear. The $O_2^{\cdot-}$-generating enzyme system of human PMNs, which is present in a dormant state in unstimulated PMNs, can be converted to an active form in a cell-free assay where both plasma membrane and cytosol fractions from resting PMNs are incubated together in the presence of the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) and either SDS or arachidonic acid (*5*). PMNs from patients with AR^+ CGD have a defect of cytosol activity in this cell-free $O_2^{\cdot-}$-generating assay, whereas the PMN membranes from these patients retain normal activity (*6*). In addition, activated PMNs from these patients do not phosphorylate a 47-kD protein, which is phosphorylated in activated PMNs from normal subjects (*7, 8*).

In the present study, PMN membrane and cytosol fractions from CGD patients were prepared (*9, 10*) and used in a fully soluble cell-free $O_2^{\cdot-}$-generating system (*11*) with modifications to adapt it to a 96-well plate microassay (*12*). When any CGD patient's PMN membranes were mixed with the same patient's PMN cytosol in this assay, there was little or no production of $O_2^{\cdot-}$. When patient PMN membranes were mixed with normal PMN cytosol and the same patient PMN cytosol was mixed with normal PMN membranes, only one of the two combinations resulted in significant $O_2^{\cdot-}$ production, depending on the type of CGD.

Six AR^+ CGD patients examined had normal activity of PMN membranes and markedly deficient activity of PMN cytosol. By contrast, eight X-linked CGD patients who were examined had significant activity in PMN cytosol and markedly deficient activity of PMN membranes, as expected for a cytochrome b_{558} defect (*2, 3, 13*). Two CGD patients, who are members of a kindred in which CGD appears to be inherited as an autosomal dominant trait (AD^+ CGD) (*1*), resembled the AR^+ CGD patients in that there was a defect in PMN cytosols but not PMN membranes.

Further studies were done to determine the nature of the cytosol abnormality in PMNs from AR^+ and AD^+ CGD patients. PMN cytosol from each of eight such patients was mixed in pairs as well as with normal cytosol in the cell-free assay containing normal PMN membranes (Fig. 1). Mixtures of each patient's PMN cytosol + normal PMN cytosol + normal PMN membranes resulted in rates of $O_2^{\cdot-}$ production similar to or greater than the rate seen with an equivalent total amount of normal PMN cytosol + normal PMN membranes, indicating that the abnormality of PMN cytosol seen in these CGD patients is not due to production of an inhibitory factor. No pairwise mixture of PMN cytosol from different AR^+ or AD^+ CGD patients results in enhancement of $O_2^{\cdot-}$ production except for those combinations that include PMN cytosol from patient J.H. (shaded panels in Fig. 1). Thus, J.H. appears to have a defect in PMN cytosol that is complementary to that seen in all of the other AR^+ CGD patients, as well as the AD^+ CGD patient (C.H.).

When normal PMN cytosol was separated on a Mono Q anion exchange column (*14*) and fractions were examined for their ability to replace cytosol in cell-free $O_2^{\cdot-}$ generation, a single major peak of activity was seen at fraction 25 (Fig. 2A). The total activity in this peak represents about 7% of the original activity applied to the column, suggesting either that activity had been destroyed during the separation or that a combination of multiple cytosol components might be required for full activity.

We added a subthreshold amount of unfractionated PMN normal cytosol to each assay mixture during a second analysis of the fractions from the Mono Q column separation in an attempt to reveal any fractions

	L.O.	P.R.	B.J.*	T.A.	R.H.	J.H.	D.C.	C.H.	S.J.*	N
L.O.	1									
P.R.	5	5								
B.J.	9	11	14							
T.A.	3	4	12	3						
R.H.	4	6	11	5	1					
J.H.	33	52	76	23	38	9				
D.C.	6	9	13	7	7	52	11			
C.H.	6	7	13	6	6	32	10	7		
S.J.	2	4	10	3	0	28	4	6	1	
N	153	122	135	141	139	142	140	139	109	82

Fig. 1. Analysis of complementation of $O_2^{\cdot-}$-generating activity in mixtures of cytosol from patients with AR^+ CGD (L.O., P.R., B.J., T.A., R.H., J.H., D.C., and S.J.) and AD^+ CGD (C.H.), as measured in the cell-free microassay (*12*). Results are expressed as change in $A_{550} \times 1000$ over 10 min, where 21 (ΔA_{550} of 0.021) equals 1 nmol of superoxide produced. For the study in each panel, PMN cytosol from the individual indicated at the left side of that row of panels was mixed with an equal amount of PMN cytosol from the individual indicated at the bottom of that column of panels, and 10^6 cell equivalents of the cytosol mixture were added to the microassay. The shaded panels indicate combinations in which complementation of different patients' PMN cytosol is evident as increased superoxide production. N, normal control; *, siblings.

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with cryptic activity that might act synergistically with the subthreshold amount of original cytosol in the generation of $O_2^{\cdot -}$ (Fig. 2A). Three additional peaks of activity were revealed at fractions 6, 56, and 69. These three fractions, which were almost devoid of activity when assayed alone, could represent individual cytosolic components required for the total activity of cytosol in the cell-free $O_2^{\cdot -}$ assay. The peak at fraction 25, by contrast, was fully competent as a replacement for normal cytosol and may represent either a complex of the essential components in fractions 6, 56, and 69 or an activated factor that did not require multiple components.

To test the hypothesis that the cryptic peaks may be individual components of a complete cytosolic system, combinations of these fractions were tested for activity in the cell-free assay in place of cytosol (Fig. 2B). Only a mixture of all three cryptic peaks resulted in substantial synergistic activation of $O_2^{\cdot -}$ production in the cell-free assay. Since some studies with the cell-free $O_2^{\cdot -}$-generating assay have reported that guanosine triphosphate (GTP) plays a regulatory role (*15*), GTP or nonhydrolyzable GTP analog was added to pair-wise mixtures of the cryptic peaks from the Mono Q column with no apparent restoration of activity (*16*). This suggests that none of the peaks was GTP. When arachidonic acid was used as the activating agent, the addition of GTP or GTP analog to the active mixture of all three peaks in the cell-free assay resulted in a twofold increase in activity when compared to arachidonic acid alone (*16*).

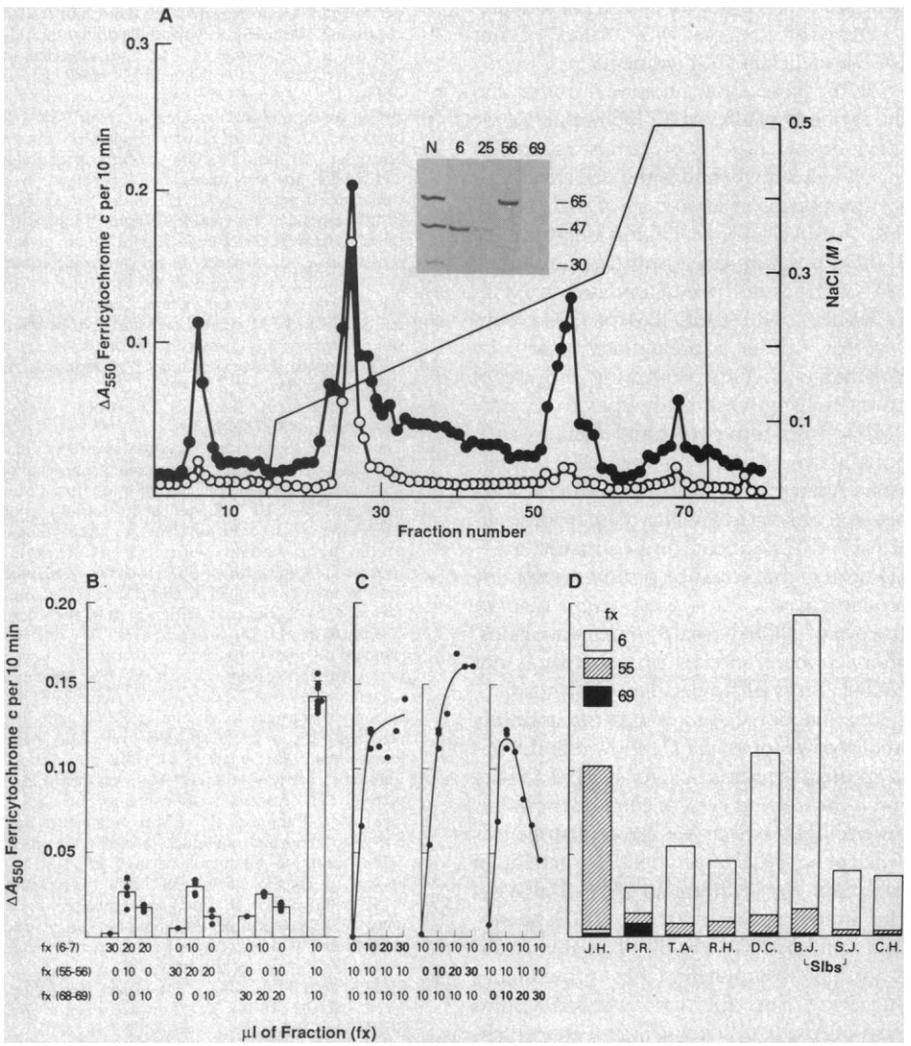

Fig. 2. Analyses of cell-free microassay $O_2^{\cdot -}$-generating activity of fractions and mixtures of fractions from a Mono Q anion exchange column separation of 5×10^9 cell equivalents of normal PMN cytosol (*14*) with or without added PMN cytosol from normal or CGD patients. In all panels the ordinate indicates ΔA_{550} as a measure of $O_2^{\cdot -}$ generation. (**A**) $O_2^{\cdot -}$-generating activity of 10-µl samples of Mono Q column fractions in the cell-free microassay in the absence (open circles) or in the presence (closed circles) of 10^5 cell equivalents of added unfractionated normal PMN cytosol. (**Inset**) SDS-PAGE immuno-peroxidase immunoblot analysis (*19*) of the indicated fractions with antibody B-1 (*17*), which detects cytosolic factors required for cell-free $O_2^{\cdot -}$ generation. (**B**) $O_2^{\cdot -}$-generating activity of different mixtures of the indicated cytosol fractions from the Mono Q column separation shown in (A). Beneath each bar is indicated the volume (in microliters) of each fraction present in the microassay. No unfractionated cytosol is present in any of the microassay samples. (**C**) Dose-response analysis of the $O_2^{\cdot -}$-generating activity of different mixtures of the indicated amounts of cytosol fractions from the Mono Q column separation shown in (A). (**D**) Ability of cytosol fractions from the Mono Q column separation to restore the defective cytosol activity of PMN from AR^+ or AD^+ CGD in the cell-free assay. Patient PMN cytosol (5×10^5 cell equivalents) was mixed in the microassay with 20 µl of the indicated fraction from the Mono Q column separation shown in (A). The results from the three different fractions tested in combination with defective cytosol from a specific patient are shown as overlapping bars; the base of each bar is at 0.

The dose-response curves for each fraction in the cell-free assay (Fig. 2C) demonstrate an optimum concentration in relation to the other components, suggesting that the stoichiometric proportions of the components are important in the activation of $O_2^{\cdot -}$ production. Each cryptic peak was then tested for the ability to restore activity to defective PMN cytosol from seven patients with AR^+ CGD and from one patient with AD^+ CGD (C.H.) (Fig. 2D). The active component in Mono Q fraction 6, which we propose calling neutrophil cytosol factor 1 (NCF-1), restored significant $O_2^{\cdot -}$-generating activity to PMN cytosol from six of seven patients with AR^+ CGD and from the one patient with AD^+ CGD. NCF-1 did not enhance the activity of PMN cytosol from patient J.H., who has AR^+ CGD. The active component in Mono Q fraction 56, which we propose calling neutrophil cytosol factor 2 (NCF-2), restored significant $O_2^{\cdot -}$-generating activity to PMN cytosol from patient J.H. but not to that of any other patient. The active component in Mono Q fraction 69, which we propose calling neutrophil cytosol factor 3 (NCF-3), was not effective at restoring activity to PMN cytosol from any of the patients tested.

Volpp *et al.* (*17*) have developed a rabbit polyclonal antibody (B-1) against a GTP-binding fraction from PMN cytosol active in the cell-free $O_2^{\cdot -}$-producing assay. On the basis of our functional studies, B-1 was used initially to perform SDS–polyacrylamide gel electrophoresis (PAGE) immunoblot analysis of PMN cytosol from S.J. and L.O., two of our AR^+ CGD patients deficient in NCF-1 activity, and J.H., our CGD patient deficient in NCF-2 activity. As reported in these studies (*17*), NCF-1 deficiency correlated with the absence of the 47-kD protein detected by this antibody in normal PMN cytosol, whereas NCF-2 deficiency was correlated with the absence of the 65-kD protein detected by this antibody. Subsequent B-1 antibody immunoblot analysis of PMN cytosol from our five other AR^+ and AD^+

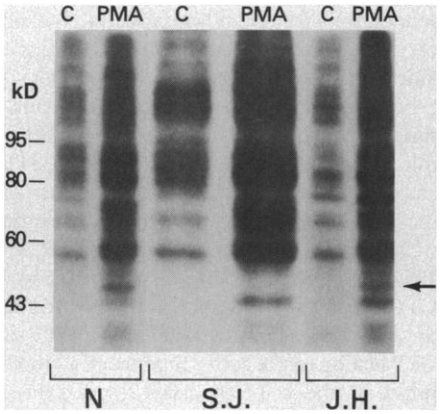

Fig. 3. Autoradiograph of SDS-PAGE separated ^{32}P-labeled phosphorylated proteins from normal PMN (N) and from PMN from patients S.J. and J.H., who have AR$^+$ CGD. Shown are control unstimulated PMN (C) and PMN activated for 5 min with PMA (1 μg/ml). The arrow indicates the position of a 47-kD phosphorylated protein present in activated PMN from N and J.H. (NCF-2 deficient), but not S.J. (NCF-1 deficient).

CGD patients showed that functional deficiency of NCF-1 activity is correlated with a specific deficiency of 47-kD protein in PMN cytosol with all cases (*16*).

We also used B-1 in SDS-PAGE immunoblot to determine the presence of these proteins in PMN cytosol fractions from the Mono Q column (Fig. 2A, inset). B-1 antibody could bind to three protein bands on immunoblot of unfractionated normal PMN cytosol at 65 kD, 47 kD, and 30 kD (lane N). NCF-1 activity in fraction 6 was correlated with the presence of only the 47-kD band as detected by this antibody. Our Mono Q column fraction 25, which can substitute for whole cytosol in the cell-free $O_2\cdot^-$ system, had some 47-kD band detectable on immunoblot. This might represent 47-kD protein in an activated state. NCF-2 activity in fraction 56 was only correlated with the presence of the 65-kD band. B-1 antibody did not react with any components from fraction 69, which contains NCF-3 activity. Instability of the activity of NCF-3 has precluded further detailed analysis, but preliminary gel filtration analysis suggests that it has a molecular mass greater than 5 kD, indicating that it is not a very low molecular mass cofactor.

Several studies have shown that phorbol myristate acetate (PMA)–activated PMNs from patients with AR$^+$ CGD do not phosphorylate a 47-kD protein that is phosphorylated in activated PMNs from normal individuals (*7, 8*). By means of a similar protocol (*7*), phosphorylation studies were done with PMNs from normal controls and from patients S.J. and J.H. Both of these patients have AR$^+$ CGD, but appear to have complementary defects of PMN cytosol as indicated in our studies above. An autoradiograph of ^{32}P-labeled control (C) and PMA-stimulated PMNs after analysis by SDS-PAGE is shown in Fig. 3. On PMA activation of normal PMNs (N), there was a marked increase in phosphorylation of a 47-kD protein. This band was absent from PMA-activated PMNs from an AR$^+$ NCF-1–deficient CGD patient (S.J.), but was clearly present in PMA-activated PMNs from an AR$^+$ NCF-2–deficient CGD patient (J.H.). The results with S.J. are representative of findings with others of our NCF-1–deficient CGD patients.

On the basis of our functional studies and the antibody studies of Volpp *et al.* (*17*), we conclude that a 47-kD protein is responsible for NCF-1 activity and is the defective cytosol component in almost all of the cases of AR$^+$ and AD$^+$ CGD. NCF-1 might be the 47-kD phosphorylation substrate in normal PMNs that is not phosphorylated in NCF-1–deficient AR$^+$ CGD PMNs. Some support for this is a preliminary report by Bolscher *et al.* (*18*), describing the use of cation exchange chromatography to separate PMN cytosol into two complementary fractions active together in the cell-free $O_2\cdot^-$ assay. A late-eluting fraction in their studies restored activity to PMN cytosol from several AR$^+$ CGD patients and contained a 47-kD protein that could be phosphorylated by protein kinase C. It is also possible that the absence of NCF-1 results in defective phosphorylation of another protein that is not NCF-1 but is of similar molecular mass.

Our functional studies and the antibody studies of Volpp *et al.* (*17*) show that the 65-kD protein is responsible for NCF-2 activity and is the missing cytosol component in our patient J.H., whom we have identified as deficient in NCF-2 activity. No patient in our study was deficient in NCF-3 activity. Our studies suggest that the CGD phenotype could occur as a result of an abnormality of this component, thus providing a theoretical basis for searching for a third form of AR$^+$ CGD involving an abnormality of NCF-3.

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9. With informed consent according to approved protocol, patient and normal volunteer peripheral blood was drawn and the PMNs separated (*1*). PMNs, treated with 5 m*M* diisopropyl fluorophosphate for 1 hour at 18°C, were washed, suspended at 10^8 cells per milliliter in Borregaard's relaxation buffer (*10*) containing 100 μ*M* phenylmethyl sulfonyl fluoride and 10 μ*M* leupeptin at 4°C, and subjected to nitrogen cavitation (*10*). Alternatively, small samples of PMN in the same buffer were sonicated twice for 5 s at conditions that resulted in about 95% cell breakage. After centrifugation (Eppendorf microcentrifuge, 10 min, 15,600*g*) to remove granules and nuclei, the supernate was centrifuged in a Beckman Airfuge (5 min, 134,000*g*) to obtain a PMN membrane pellet and PMN cytosol supernate. Membranes were extracted in 1.16% sodium deoxycholate in a buffer system as previously described (*11*), resulting in a final concentration of 5×10^8 cell equivalents of solubilized membrane per milliliter. Solubilized PMN membrane and PMN cytosol were stored at −70°C without loss of activity.
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12. The cell-free superoxide generation assay was done in a 96-well flat bottom microplate (Immulon 1, Dynatech) by addition of 10 μl of solubilized PMN membrane (5×10^5 cell equivalents) and 10 μl of PMN cytosol (10^6 cell equivalents) to a final volume of 100 μl of reaction buffer (0.2 m*M* ferricytochrome c, 4 m*M* $MgCl_2$, 10 μ*M* flavin adenine dinucleotide, 1 m*M* EGTA, 100 μ*M* NADPH, and 100 μ*M* SDS, buffered with 75 m*M* potassium phosphate at *p*H 7.0) in each well. This was the standard microassay condition with samples assayed in quadruplicate except where noted. The plate was shaken for 30 s at 22°C on a Vortex Genie 2 (Fisher Scientific), warmed for 1 min at 37°C, and then placed at 22°C in the plate holder of a Microplate Reader MR 600 (Dynatech) to follow the reaction. Light absorbance at 550 nm (A_{550}) was determined every 5 min. Control wells contained 2.5 μg of superoxide dismutase. For PMN membranes and cytosol from normal individuals (n = 12), the superoxide dismutase inhibitable change in A_{550} was linear over the first 15 min, averaging 0.090 ± 0.005 per 10 min, and represented 4.2 nmol of superoxide per 10 min. No superoxide generation was detected when either membrane or cytosol was left out of the assay.
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14. The proteins that precipitated from normal PMN cytosol between 30 and 55% saturated ammonium sulfate at 4°C were resuspended in 1/10 volume of 20 m*M* tris buffer (*p*H 7.5) containing 10% w/v betaine (Sigma), and were centrifuged; the supernate was desalted over a Sephadex G-25 column into the same buffer, resulting in a concentrated cytosol fraction containing greater than 40% of the original activity of cytosol. This fraction was applied to a Mono Q MR 10/10 anion exchange column (Pharmacia) and eluted with an NaCl gradient (0 to 0.5*M*) in the same buffer with the use of a Gilson high-pressure liquid chromatography (HPLC) system (flow rate, 2 ml/min). Fractions of 1.1 ml were collected and desalted over a Sephadex G-25 column into cell-free assay buffer (*12*) without NADPH or SDS.
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Reversal of Chloroquine Resistance in Malaria Parasite *Plasmodium falciparum* by Desipramine

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Desipramine and several other tricyclic antidepressant drugs reverse chloroquine resistance in *Plasmodium falciparum* in vitro at concentrations observed in the plasma of human patients treated for depression. Reversal of resistance is associated with increased chloroquine accumulation in the parasite, probably because of inhibition of a putative chloroquine efflux pump. When owl monkeys (*Aotus lemurinus lemurinus*) infected with chloroquine-resistant *Plasmodium falciparum* were treated with chloroquine plus desipramine, their parasitemias were rapidly suppressed. Desipramine was found to be one of the most effective compounds yet described for the reversal of chloroquine resistance both in vitro and in vivo.

CHLOROQUINE, A 4-AMINOQUINOline introduced for the treatment of malaria over 40 years ago (*1*), is highly effective against susceptible strains of *Plasmodium falciparum*; however, chloroquine resistance, which was first reported in 1961 (*2*), now occurs in most geographic regions where malaria is endemic. It is now known that resistant plasmodia accumulate less chloroquine than do susceptible strains (*3*), and recent work shows that this reduced accumulation may be due to rapid efflux of the drug from the resistant parasite. Verapamil, a calcium channel blocker, inhibits this process (*4*) and reverses chloroquine resistance in *P. falciparum* in vitro (*5*). Other calcium antagonists reverse chloroquine resistance in vivo (*6*).

The knowledge that some tricyclic psychotropic drugs have weak intrinsic antimalarial activity (*7*) and are calcium antagonists (*8*) prompted us to study one class of these drugs in combination with chloroquine. We report here that desipramine (Norpramin), as well as other tricyclic antidepressant compounds, reverses chloroquine resistance in two *P. falciparum* strains in vitro at concentrations that occur in the plasma of patients undergoing treatment for depression (*9*). Owl monkeys (*Aotus lemurinus lemurinus*) infected with chloroquine-resistant *P. falciparum* and treated with desipramine plus chloroquine showed rapidly suppressed parasitemias, demonstrating the potential clinical use of this chemotherapeutic approach.

Three strains of *P. falciparum* were used: chloroquine-susceptible West African clone D-6 (*10*) [chloroquine IC_{50} (50% inhibitory concentration) = 7 ng/ml], chloroquine-resistant FCR-3 (*11*) (chloroquine IC_{50} = 70 ng/ml), and the multidrug-resistant Indochina clone W-2 (*10*) (chloroquine IC_{50} = 160 ng/ml). The parasites were maintained in human erythrocytes (type 0+; 6% hematocrit) in vitro in RPMI 1640 medium and 10% human serum (*12*). Drug testing was carried out by following [^{3}H]-hypoxanthine incorporation to measure growth rates of *P. falciparum* in the semiautomated microdilution technique (*13*) in which hematocrits were 1% and starting parasitemias were 0.5%. *P. falciparum* was incubated with test compounds for 48 hours.

Evidence for marked synergism between desipramine and chloroquine against chloroquine-resistant *P. falciparum* (FCR-3) was obtained when we used isobologram analysis (*5*). Quantitative analysis of the efficacy of the desipramine-chloroquine combination was done by constructing dose-response curves for chloroquine in the presence of several fixed concentrations of desipramine. The presence of 20 to 500 ng of desipramine per milliliter caused a shift of the dose-response curves to the left, showing that desipramine produced reversal of chloroquine resistance in the multidrug-resistant Indochina W-2 clone (Fig. 1A). Similar results were obtained with the FCR-3 strain. Desipramine did not change the response of the chloroquine-sensitive West African clone D-6 to chloroquine (Fig. 1B). Desipramine alone at 500 ng/ml did not inhibit the growth of *P. falciparum* (FCR-3) by more than 20 to 30% (IC_{50} > 4000 ng/ml). A number of antidepressant drugs were analyzed in this manner, and the concentrations of drug that would lower the chloroquine IC_{50} by 50% and 80% were determined (Table 1).

Desipramine increased the accumulation of [^{3}H]chloroquine by approximately ten times in clone W-2 and approximately three

Table 1. Reversal of chloroquine resistance with antidepressant drugs. The parasites were incubated with serial twofold dilutions of chloroquine known to produce well-defined dose-response curves along with fixed concentrations of antidepressant drugs. [^{3}H]Hypoxanthine incorporation was used as an indicator of antimalarial effects in vitro. The values represent the mean concentrations of antidepressant drugs that cause either a 50% or an 80% decrease in the IC_{50} of chloroquine in at least two experiments in which the results were within 30% of the mean.

Drug	Concentration of drugs needed for reversal of chloroquine resistance (ng/ml)			
	West African FCR-3 strain		Indochina W-2 clone	
	50%	80%	50%	80%
Desipramine	40	150	35	120
Protriptyline	30	80	50	160
Imipramine	30	130	50	150
Nortriptyline	40	120	80	200
Doxepin	70	150	35	140
Amoxapin	120	320	360	>500
Maprotiline	165	500	280	>500
Mianserin	350	>500	>500	>500
Trazodone	>500	>500	>500	>500

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