$$\begin{array}{ccc} & {}^{0}\Delta G_{diss} & \\ T & \rightleftharpoons & 2D \\ {}^{T}\Delta G_{ox} \downarrow\uparrow & & \downarrow\uparrow 2^{D}\Delta G_{ox} \\ T(O_2)_4 & \rightleftharpoons & 2D(O_2)_2 \\ & {}^{4}\Delta G_{diss} & \end{array} \quad (3)$$

The symbols represent Hb dimer (D) and tetramer (T) species, with liganding by $O_2$. (Hb dimer is the noncooperative two-subunit $\alpha_1\beta_1$ half-molecule.) The equilibrium free energy for $O_2$ binding is indexed to the dimer (${}^{D}\Delta G_{ox}$) or tetramer (${}^{T}\Delta G_{ox}$). The free energy for subunit dissociation is indexed to unliganded (${}^{0}\Delta G_{diss}$) or four-liganded (${}^{4}\Delta G_{diss}$) Hb. The $\Delta G$ values are defined in Eq. 3 from left species to right and from top species to bottom. The cooperative free energy, $\Delta G_{coop}$, is the difference in energy for $O_2$ binding to a cooperative hemoglobin tetramer instead of two noncooperative dimers (Eq. 4).

$$\Delta G_{coop} \equiv {}^{T}\Delta G_{ox} - 2\,{}^{D}\Delta G_{ox} = {}^{0}\Delta G_{diss} - {}^{4}\Delta G_{diss} \quad (4)$$

An analogous equation can be written for any modified Hb. The change in cooperative free energy due to the modification ($\delta\Delta G^{mod}{}_{coop}$) can then be obtained by subtracting the two equations (Eq. 5).

$$\delta\Delta G^{mod}{}_{coop} = \delta^{T}\Delta G_{ox} - 2\delta^{D}\Delta G_{ox} = \delta^{0}\Delta G_{diss} - \delta^{4}\Delta G_{diss} \quad (5)$$

All of the equations used here are written to make loss in allosteric cooperativity and in structural stability appear as positive free energy changes.

The term $\delta\Delta G^{mod}{}_{coop}$ was obtained from $O_2$-binding curves according to Eq. 6 (*9*), which connects the change in cooperative free energy to the median partial pressure measured for unmodified Hb ($P_{m,HbA}$) and modified Hb ($P_{m,mod}$) [at 5.5° ± 0.5°C in a Gill apparatus; (*14*)].

$$\delta\Delta G^{mod}{}_{coop} = -4RT\ln(P_{m,mod})/(P_{m,HbA}) \quad (6)$$

Here $\delta\Delta G^{mod}{}_{coop}$ is formally equal only to $\delta^{T}\Delta G_{ox}$ in Eq. 5 (*9*). However, the term $2\delta^{D}\Delta G_{ox}$ is normally zero, since the Hb dimer (half-molecule) is noncooperative and is unaffected by allosteric modifications. Accordingly, the $\delta\Delta G$ values from Eq. 6, listed in Table 1 under "$O_2$ binding," can be directly compared to the subunit dissociation values obtained from experimental measurement of the last two terms in Eq. 5. This comparison is shown in the two columns at the right of Table 1. Modification-specific free energies obtained from changes in HX rate were measured in deoxy Hb (Eq. 2b); thus, they are more properly compared to the term for dissociation of unliganded Hb, $\delta^{0}\Delta G_{diss}$. This comparison is shown in the two left columns of Table 1.

14. S. J. Gill, E. DiCera, M. L. Doyle, G. A. Bishop, C. H. Robert, *Biochemistry* **26**, 3995 (1987).
15. R. S. Molday, S. W. Englander, R. G. Kallen, *ibid.* **11**, 150 (1972).
16. We thank M. R. Busch and C. Ho for their generous gift of desHis hemoglobin. Supported by NIH research grants to S.W.E., G.K.A., and S.J.G. This paper is dedicated to the memory of Stanley Jensen Gill. He has enriched our science and our lives.

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# Vaccine Protection of Chimpanzees Against Challenge with HIV-1–Infected Peripheral Blood Mononuclear Cells

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**Because human immunodeficiency virus (HIV) can be transmitted as cell-free virus or as infected cells (cell-associated virus), vaccines must protect against infection by both viral forms. Vaccine-mediated protection of nonhuman primates against low doses of cell-free HIV-1, HIV-2, or simian immunodeficiency virus (SIV) has been demonstrated. It is now shown that multiple immunizations of chimpanzees with HIV-1 antigens protected against infection with cell-associated virus. Protection can persist for extended periods (one animal had not been exposed to viral antigens for 1 year before challenge). These results show that it is possible to elicit long-lasting protective immunity against cell-associated HIV-1.**

Irrespective of the stage of infection or disease, persons infected with HIV have both virus-infected cells (cell-associated virus) and cell-free virus (*1*). Transmission of HIV can occur with either or both forms of virus, especially through exchange of blood, as occurs among intravenous drug abusers. Although data regarding the quantity and primary form of HIV in vaginal and seminal fluids are limited (*2*), it can be assumed that both cell-free and cell-associated viruses are also transmitted through sexual contact. Therefore, any effective vaccine against HIV must protect against both viral forms.

Infection of either chimpanzees with HIV-1 or macaque species with HIV-2 or SIV has been used to show that vaccination can elicit protection against challenge with these lentiviruses (*3–5*). In all cases, however, protection was demonstrated against low doses of infectious cell-free virus. We show that immunized chimpanzees were protected against intravenous challenge with peripheral blood mononuclear cells (PBMC) from an HIV-infected chimpanzee.

Chimpanzee C-339 was immunized with various HIV-1 antigens (Table 1) and was subsequently challenged with an intravenous injection of 100 median tissue culture infectious dose ($TCID_{50}$) of cell-free HIV-1 (*4, 6*). This animal remained virus negative by multiple criteria and did not develop an anamnestic antibody response to HIV through 40 weeks after challenge. Because in vivo studies had indicated that immune stimulation induced increases in HIV-1 expression in infected chimpanzees (*7*), and to ensure that C-339 had indeed been protected from infection, we attempted to induce detectable expression of putative latent virus by stimulating the animal's immune system. At week 40 after challenge, C-339 was inoculated with the Syntex adjuvant formulation, SAF-1, and at weeks 44 and 48, with a mixture of HIV-1 antigens (*8*). None of these inoculations resulted in detection of the virus, as indicated by cocultivation of C-339's PBMC with normal human PBMC. The injections of HIV-1 antigens, however, served as booster immunizations, and increases in both total antibodies to HIV-1 (Fig. 1) and neutralizing antibody titers (twofold) were observed.

At week 52, C-339 and a control chimpanzee, C-435, were challenged intravenously with HIV-1–infected cells. The challenge inoculum consisted of cryopreserved PBMC obtained from a chimpanzee, C-087, 14 weeks after infection [positive control in another vaccine study (*4*)] with $HIV\text{-}1_{Lai}$ (HTLV-IIIB strain). Although it is possible that the PBMC inoculum may have contained some cell-free virus, it is likely that few, if any, virions were present as a result of its preparation (*9*). Because the minimal infectious dose of HIV-infected cells required for infection of chimpanzees has not been determined, and because of the limited number of available chimpanzees, the challenge dose was selected empirically. This selection was based on the results of multiple in vitro titrations (*7*) of aliquots of the cryopreserved PBMC from C-087. Because there has been good agreement between in vitro $TCID_{50}$ determinations for cell-free virus and 50% animal infectious doses for both HIV-1 infectious stocks for chimpanzee inoculations and a majority of SIV stocks used to inoculate macaques, there is no reason to believe that infected cells would give substantially dif-

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ferent results. In fact, one would assume that ten HIV-infected cells would be more infectious than ten virions because even one cell actively producing virus has the potential to produce several $\log_{10}$ virus particles. If one assumes that one infected cell is sufficient for a culture to become virus positive, then the PBMC titrations indicated that there was a mean of 250 infectious cells (range of 1 SD, 161 to 385 cells) per $10^7$ total PBMC (*10*). Chimpanzees C-339 and C-435 were inoculated intravenously with a 1-ml suspension, which contained $5.8 \times 10^5$ viable PBMC or 15 infectious PBMC (range of 1 SD, 9 to 22 cells); this dose reflects a minimum number of infectious cells present.

After inoculation, the animals were observed daily, and blood samples were obtained every 2 weeks for a period of 8 weeks and at monthly intervals thereafter. Virus isolation attempts were performed by cocultivation of PBMC with phytohemagglutin (PHA)-stimulated normal human PBMC, as described (*4*). We also attempted to isolate virus from bone marrow aspirates obtained at 3 and 9 months and from lymph node biopsies at 6 and 11 months after inoculation. At 4 weeks after challenge and at every time thereafter, virus was isolated from PBMC as well as bone marrow and lymph node samples from the control C-435. In contrast, virus was not isolated from PBMC, bone marrow, or lymph node biopsies from the immunized chimpanzee, C-339. Polymerase chain reaction (PCR) analyses with both *env* and *pol* primers (*4, 11, 12*) of DNA isolated from C-339's PBMC and bone marrow aspirates were consistently negative. HIV-specific antibodies were detected in serum from C-435 initially at 8 weeks after challenge, and titers continued to rise through week 24 (Fig. 1). However, no anamnestic response was detected in serum from C-339, and antibody titers to HIV-1 diminished slightly, then remained stable.

At the time of challenge, C-339's serum contained neutralizing activity against cell-free HIV-1. We also tested in vitro whether C-339's serum could interfere with transmission of virus from PBMC from an HIV-1–infected chimpanzee to normal human PBMC. Compared to serum obtained from C-339 before immunization, which had no inhibitory activity, serum from weeks 0 and 52 inhibited virus transmission and production by 68 and 75%, respectively (Fig. 2). Serum from week 24 showed less inhibition, which probably reflects a gradual loss of activity after the initial virus challenge. The subsequent increase in inhibitory activity at week 52 resulted from the HIV-1 booster injections that C-339 received at weeks 44 and 48.

After the apparent success at preventing infection of C-339 by cell-associated HIV-1, two additional immunized chimpanzees were challenged with an equivalent number of infectious cells from the same cryopreserved PBMC stock from C-087 (Table 1). One chimpanzee, C-499, which had been immunized, challenged with cell-free HIV-1, and remained virus negative for 1 year (*4*), was not reimmunized before challenge with HIV-infected PBMC. The second animal, C-447, had not been exposed previously to infectious HIV-1.

After challenge, these two chimpanzees were monitored in the same manner as C-339. HIV-1 antibody titers in both animals remained stable, and virus was not isolated from any of the blood or tissue samples. At 7 months after challenge, C-499 died as a result of congestive heart failure. Peripheral blood and bone marrow cells, as well as minced tissue fragments from brain, spleen, lymph nodes, kidney, liver, and salivary gland, were cocultivated with human PBMC. All cultures were monitored for 6 weeks and remained negative for reverse transcriptase activity. DNA extracted from these tissue samples on three separate occasions, in two different laboratories, was subjected to conventional and nested PCR analysis a total of six times with either *env* or *gag* primers (*4, 12, 13*). None of the tissues was positive.

All PBMC, bone marrow, and lymph node samples from the other animal, C-447, remained negative for virus by cocultivation and PCR analysis through 12 months of follow-up. Thus, three of three

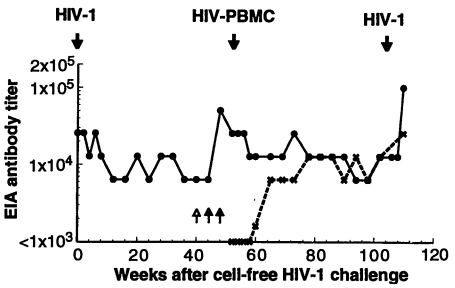


**Fig. 1.** HIV-1–specific antibody titers in serum from C-339 (●) and C-435 (X). Titers are given as the last serum dilution that was positive in an HIV-1 whole virus EIA kit (Genetic Systems, Seattle, Washington). Week 0 is the time at which C-339 was first challenged with cell-free HIV-1. The small open arrow indicates the time at which C-339 was inoculated with SAF-1 adjuvant, and the two small closed arrows indicate times at which C-339 received injections of HIV-1 antigens formulated with SAF-1. HIV-1, times at which C-339 was challenged with cell-free HIV-1; HIV-PBMC, time at which both C-339 and C-435 were challenged with cell-associated HIV-1 (PBMC from C-087).

**Table 1.** Immunization history of chimpanzees prior to challenge with HIV-infected cells.

| Animal | Immunization* | Prior challenge with cell-free HIV-1 | Status at time of cell-associated challenge | | |
|---|---|---|---|---|---|
| | | | EIA anti-HIV titer† | Neutralizing titer‡ | Virus recovery§ |
| C-339 | Inactivated HIV gp160, V3-KLH | Yes | 25,600 | 1:64 | None |
| C-435 | None | No | <100 | <4 | Week 4 |
| C-499 | gp160, p18, 21 V3 peptides | Yes | 6,400 | 1:512 | None |
| C-447 | gp160, p18, p25, vif, nef, V3 peptide | No | 6,400 | 1:256 | None |

*Immunogens and regimen of inoculation for C-339 and C-499 are described in (*4*). Originally in parallel with C-499, C-447 was immunized intramuscularly with a mixture of HIV-$1_{Lai}$ gp160*env*, p18*gag*, *vif*, and *nef* proteins (125 to 150 μg each per dose), formulated in SAF-1 and administered seven times over 15 months. After a 13-month rest period, C-447 was given a booster immunization of 150 μg each of purified gp160*env* and p18*gag* in SAF-1. This was followed 4 months later by three injections at monthly intervals of a mixture of *nef* gene and V3 peptides from the human T cell lymphotropic virus (HTLV)-IIIB strain. The *nef*-encoded peptide represented a CTL epitope in this protein (*20*). Challenge with cell-associated HIV-1 was 11 weeks after the last injection of peptides. †EIA titers are the reciprocal of the highest dilution of serum that gave a positive result using the Genetics Systems HIV-1 EIA kit. ‡Neutralizing antibody titers were determined by inhibition of syncytia formation in CEM-SS cells as described (*21*). §Attempts at virus recovery from PBMC were by cocultivation with normal human PBMC biweekly for 8 weeks and then monthly. For C-435, time at which virus was first recovered from PBMC.

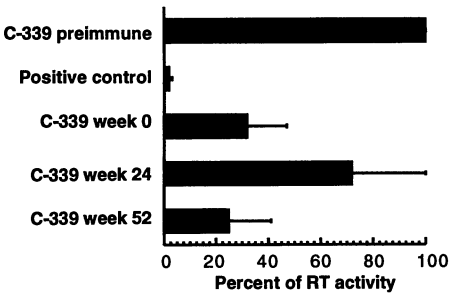


**Fig. 2.** Inhibitory effects of serum from C-339 on cell-to-cell transmission of HIV-1. Results are the averages of three experiments, performed as described (*19*), and are depicted as percent of RT activity in wells containing preimmune serum from C-339 (on day 15). Error bars represent 1 SD.

immunized chimpanzees were apparently protected (as assessed by conventional criteria) from infection by HIV-1–infected cells.

At the time of challenge with HIV-infected PBMC, C-447 and C-499 had lower HIV-1 enzyme-linked immunosorbent assay (EIA) antibody titers but substantially higher neutralizing antibody titers compared with those of C-339 (Table 1). Serum samples obtained from C-447 and C-499 on the day of challenge were tested in vitro and found to inhibit cell-to-cell transmission of HIV-1 by 25 and 52%, respectively. Although these levels were less than the 75% inhibition observed with serum from C-339 on the day this animal was challenged, it is possible that this activity contributed to the protective response. Whether this assay is a reliable predictor of protection against cell-associated virus challenge cannot be determined with these limited data.

When C-339 had been virus free for 1 year after the cell-associated HIV-1 challenge (week 104 relative to the initial cell-free virus challenge), the animal was inoculated again with a dose of cell-free HIV-1 equivalent to that used for the first challenge (*4*, *14*). HIV-1 was first detected in PBMC from C-339 at 4 weeks, and an increase in HIV-1 EIA antibody titer was observed at 6 weeks after challenge (Fig. 1). C-339 had not been exposed to HIV-1 antigens during the 12 months before this second challenge with cell-free HIV-1. Thus, it appeared that the immune response elicited by vaccination did not persist at a level sufficient to protect against this last exposure to virus. C-339 became infected despite the presence of an apparently stable humoral response, which shows that this animal was not inherently resistant to HIV-1 infection.

The mechanism of protection of the three chimpanzees against challenge with HIV-infected cells is not known. Because C-087 and the three chimpanzees that were challenged with HIV-infected PBMC from C-087 were not siblings, the possibility that the four animals shared identical major histocompatibility complex (MHC) haplotypes is low. Thus, one would assume a priori that the initial protection against C-087's PBMC was not mediated by classical MHC-restricted cytotoxic T lymphocyte (CTL) activity. We have been unable to detect CTL activity directly in peripheral blood lymphocytes from immunized chimpanzees. If CTLs are present, they must reside predominantly in other lymphoid tissues, and their frequency in the circulating pool of PBMC is very low or nonexistent. As another measure of cell-mediated immunity, PBMC obtained from all three chimpanzees on the day of cell-associated virus challenge were tested in vitro for the ability to prevent cell-to-cell transmission. Although some inhibitory activity was detected, results were inconsistent (data not shown), and this assay did not correlate with protection in vivo.

More likely mechanisms of protection might be antibody-dependent cellular cytotoxicity (ADCC), previously detected in serum from the three immunized animals (*15*), and antibody complement–mediated cytotoxicity, which has been reported in HIV-1–infected chimpanzees (*16*). These animals had antibodies that neutralized cell-free virus and partially inhibited cell-to-cell transmission. Some of this latter activity may have been due to antibodies to the V3 loop which inhibited cell fusion. Such antibodies have been shown to protect chimpanzees from infection with cell-free HIV-1 after passive antibody transfer (*17*). It is probable, however, that both HIV-specific antibodies and cell-mediated activities synergized to effect protection.

The protection observed here is most likely different from that associated with anti-cell activity reported to mediate protection in macaques immunized against SIV (*18*). When serum from the chimpanzees was tested for the presence of antibodies to human cellular antigens, only serum from C-339 was positive. This is consistent with the observation that C-339 was immunized with whole inactivated HIV, whereas C-447 and C-499 were vaccinated only with purified antigens or antigens expressed by recombinant vaccinia viruses.

Ideally, a vaccine should be one that elicits long-lasting immunity after a minimal number of immunizations. Although we have observed long-lasting, stable EIA and neutralizing antibody titers in our immunized chimpanzees, these were achieved with a large number of immunizations over a 2-year period. These regimens are not practical for use in human populations. Although progress in HIV-1 vaccine development has been made (*3*–*5*), a continuing challenge is to define mechanisms by which immunization blocks experimental infection and establishment of virus load.

## REFERENCES AND NOTES


1. D. D. Ho, T. Moudgil, M. Alam, *N. Engl. J. Med.* **321**, 1621 (1989); R. W. Coombs *et al.*, *ibid.*, p. 1626.
2. J. A. Levy *et al.*, *Ann. Intern. Med.* **103**, 694 (1985); M. W. Vogt *et al.*, *Lancet* i, 525 (1986); C. B. Wofsy *et al.*, *ibid.*, p. 527; M. S. Borzy, R. S. Connell, A. A. Kiessling, *J. AIDS* **1**, 419 (1988).
3. P. W. Berman *et al.*, *Nature* **345**, 622 (1990).
4. M. Girard *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 542 (1991).
5. R. C. Desrosiers *et al.*, *ibid.* **86**, 6353 (1989); M. Murphey-Corb *et al.*, *Science* **246**, 1293 (1989); J. R. Carlson *et al.*, *AIDS Res. Hum. Retroviruses* **6**, 1239 (1990); E. J. Stott *et al.*, *Lancet* ii, 1538 (1990); P. Putkonen *et al.*, *AIDS Res. Hum. Retroviruses* **7**, 271 (1991); A. Shafferman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7126 (1991); P. R. Johnson *et al.*, *ibid.* **89**, 2175 (1992).
6. All chimpanzees were adult males previously used in hepatitis experiments. They were maintained in biosafety level 2 and 3 facilities at the Laboratory for Experimental Medicine and Surgery in Primates, and experimental procedures were performed according to accepted practices for the care and use of primates in biomedical research.
7. P. N. Fultz, J.-C. Gluckman, E. Muchmore, M. Girard, *AIDS Res. Hum. Retroviruses* **8**, 313 (1992). In the assays to quantitate numbers of infectious cells in C-087's PBMC, PHA-stimulated normal PBMC from both humans and chimpanzees were used as indicator cells.
8. The mixture of antigens described in (*4*) was formulated with SAF-1 and included inactivated HIV-$1_{Lai}$, recombinant antigens gp160*env* and p25- and p18-*gag*, and peptides representing the V3 immunodominant loop.
9. Heparinized blood was obtained from chimpanzee C-087, and PBMC were purified by centrifugation through lymphocyte separation medium (Organon Teknika, West Chester, PA). The cells were washed twice and cryopreserved in multiple vials in RPMI-1640 medium containing 25% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). Immediately before inoculation, PBMC in one vial were rapidly thawed and washed; the cells were counted, diluted in RPMI-1640, and injected into the animals within minutes. Previous studies in our laboratory and by others have shown that one freeze-thaw cycle does not increase the apparent number of HIV-1–infected PBMC.
10. The mean number of infectious cells was calculated with the use of the VacMan Program designed by J. L. Spouge, National Library of Medicine, National Institutes of Health, Washington, DC. At 95% confidence, the inoculum contained from 6 to 33 HIV-1–infected PBMC.
11. F. Laure *et al.*, *Lancet* ii, 538 (1988).
12. P. Simmonds *et al.*, *J. Virol.* **64**, 864 (1990).
13. Nested primers for the HIV-1 *gag* gene were 881 and 882 for the first round of amplification and 883 and 990 for the second round of amplification, as described by P. Simmonds *et al.* (*12*).
14. A cryopreserved aliquot of the same virus stock (obtained from Larry Arthur, National Cancer Institute–Frederick Cancer Research Facility) was used; 100 $TCID_{50}$ were injected intravenously in a total volume of 1 ml.
15. M. Belo *et al.*, *AIDS* **5**, 169 (1991).
16. P. L. Nara *et al.*, *J. Virol.* **61**, 3173 (1987).
17. E. A. Emini *et al.*, *ibid.* **64**, 3674 (1990).
18. E. J. Stott, *Nature* **353**, 393 (1991); A. J. Langlois, K. J. Weinhold, T. J. Matthews, M. L. Greenberg, D. P. Bolognesi, *Science* **255**, 292 (1992).
19. Serum samples (50 μl) were placed in duplicate wells of 24-well plates, which contained $3 \times 10^5$ PBMC from an HIV-1–infected chimpanzee (C-527) in 0.5 ml of medium. (Because of limited numbers of cryopreserved PBMC from C-087, PBMC from C-527, an animal infected at the same time as C-087 and with the same HIV-1 strain, were used.) After 1 hour of incubation at 37°C, an equal number of PHA-stimulated human PBMC was added to each well. Samples (1 ml) of cell-free medium were removed on days 6, 10, and 14 or 15, centrifuged to pellet virus, and reverse transcriptase (RT) assays performed. The medium was replaced at the time samples were removed to maintain volumes of 2.5 ml; an additional 10 μl of undiluted serum was added on day 6 only. As a positive control, serum from an HIV-1–infected chimpanzee that completely inhibited cell-to-cell transmission (P. N. Fultz, unpublished data) was always included. HIV-infected PBMC cultured alone did not result in detectable virus.
20. B. Culmann *et al.*, *J. Immunol.* **145**, 1560 (1991); F. Hadida *et al*, *J. Clin. Invest.* **89**, 53 (1992).
21. P. L. Nara *et al.*, *AIDS Res. Hum. Retroviruses* **3**, 283 (1987).

22. We thank J. Stallworth, P. May, N. Dunlop, and C. Calvano for technical assistance; A. Langlois for detection of antibodies to cell-surface antigens; A. Pinter for performing confirmatory neutraliza-

tion assays; G. Ferrari and E. Gomard for efforts to detect CTL; and J.-P. Levy for assistance. We appreciate the continuous support of J.-P. Lecocq and regret his untimely death. Supported by Pasteur Merieux Serums et Vaccins and the French National AIDS Research Agency (ANRS). M.L.G. is an American Foundation for AIDS Research–Michael Bennett Scholar.

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# Myoglobin in a Cyanobacterium

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**Myoglobin was found in the nitrogen-fixing cyanobacterium *Nostoc commune*. This cyanobacterial myoglobin, referred to as cyanoglobin, was shown to be a soluble hemoprotein of 12.5 kilodaltons with an amino acid sequence that is related to that of myoglobins from two lower eukaryotes, the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*. Cyanoglobin is encoded by the *glbN* gene, which is positioned between *nifU* and *nifH*—two genes essential for nitrogen fixation—in the genome of *Nostoc*. Cyanoglobin was detected in *Nostoc* cells only when they were starved for nitrogen and incubated microaerobically.**

Cyanobacteria may have figured in the evolution of Earth's oxygenic atmosphere (*1*). Many cyanobacteria have nitrogenase, an enzyme that catalyzes the reduction (fixation) of dinitrogen; as a group, these photosynthetic microorganisms are responsible for much biological dinitrogen fixation (*2*). Nitrogenase is sensitive to gaseous oxygen, and cyanobacteria use various mechanisms to achieve aerobic nitrogen fixation, including attenuation of the oxygen tension in the immediate vicinity of nitrogenase. For example, some cyanobacteria can effect either a spatial or temporal separation, or both, of their oxygen-evolving and nitrogen-fixing activities (*3*, *4*), whereas others restrict their nitrogenase activity to a specialized differentiated cell, the heterocyst. During the course of its differentiation, the heterocyst is modified structurally and biochemically in order to provide a reducing environment conducive to nitrogen fixation (*5*). Marked changes in gene expression accompany heterocyst differentiation and include the induction of the *nif* operons (*6*).

Of the heterocystous cyanobacteria, species of *Nostoc* are especially prevalent in terrestial environments from the tropics to the polar regions, where they often enter into associations with a variety of higher and lower plants (*7*). One of these species, *Nostoc commune*, forms visually conspicuous colonies in the shallow natural depressions of limestone rock in karst areas. We isolated the *nifUHD* gene cluster from the strain *Nostoc commune* UTEX 584 (*8*). DNA sequence analysis revealed an open reading frame (ORF) of 118 codons, potentially encoding a 12.5-kD protein, between *nifU* and *nifH* that showed no obvious similarity in either DNA or amino acid sequence with any known *nif* genes or Nif proteins, respectively (Fig. 1A). However, marked similarity was apparent, both in size and in amino acid sequence, to the myoglobins of the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*, which contained 116 and 121 amino acids, respectively (*9*, *10*). We used the polymerase chain reaction (PCR) (*11*) to amplify *glbN*, subcloned *glbN* into an expression vector (pT7-7), and overexpressed the recombinant protein in *Escherichia coli* (Fig. 1A) (*12*). A 12-kD protein was revealed by electrophoresis of whole-cell lysates from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced (Fig. 1B, lanes C and D) cells carrying the cloned *Nostoc* gene (*13*); the protein was not revealed in cells uninduced by IPTG (Fig. 1B). The cell pellets from GlbN$^+$ transformants were bright red in color. Examination of induced cells with light microscopy did not reveal inclusion bodies.

The recombinant protein partitioned to the soluble fraction after disruption of GlbN-expressing *E. coli* in a French pressure cell. During the course of the purification, the color of the protein preparation changed from bright red to orange-brown, probably the result of auto-oxidation of the protein to the ferric (met)-form. This is a characteristic of myoglobins and hemoglobins isolated from other sources. The orange-brown fraction, obtained after ion-exchange chromatography, ultrafiltration, and gel-exclusion chromatography, was judged to be homogenous when examined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 1B). The molecular sizes of GlbN estimated from gel filtration and SDS-PAGE were 15 kD and 12 kD, respectively. These data indicate that recombinant GlbN is a monomer.

The ultraviolet (UV)-visible spectrum of the recombinant GlbN was consistent with that of a high-spin ferric hemoprotein, with a maximum absorbance in the Soret region at 412 nm (Fig. 2). Reduction with sodium dithionite shifted the Soret peak to 422 nm, whereas addition of carbon monoxide to the reduced protein caused a shift in the Soret peak to 419 nm. These data demonstrate that the reduced protein has an open ligand position that can bind carbon monoxide and probably molecular oxygen. As no cysteine residues are present in

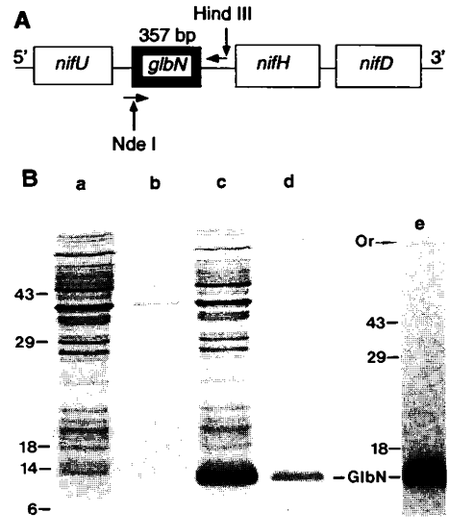


**Fig. 1.** Chromosomal location of *glbN* and purification of recombinant GlbN protein. (**A**) The position of *glbN* in the *nifUHD* cluster of *Nostoc commune* (*8*) and the region amplified by PCR are represented schematically. Note that the *nifU-nifH* intergenic region of the related cyanobacterium *Anabaena sp.* strain PCC7120 is devoid of any ORF (*22*). The two oligonucleotides used were 5′-CTGGCATATGAGCACATTGTACG-3′ and 5′-GCGAAGCTTCGATAGGACTTGAT-3′. These are complementary to the 5′ end of *glbN* and to a region 50 bp downstream of its 3′ terminus, respectively. Recognition sites for Nde I and Hind III were introduced into the oligonucleotides to permit subsequent subcloning of the PCR product in the desired orientation in pT7-7. The sequence of *glbN* is 357 bp; the engineered PCR product was 422 bp. The first five amino acid residues of GlbN—represented by bases 8 to 22 in the first oligonucleotide—are Met, Ser, Thr, Leu, and Tyr. The sequence of *glbN* has been submitted to GenBank (accession number M92437). (**B**) Lysates from uninduced (lanes a and b) and induced cells (lanes c and d); purified GlbN prepared by liquid chromatography and examined by SDS-PAGE (lane e). Lanes b and d were loaded with one-tenth the amount of total protein present in lanes a and c. Molecular sizes in kilodaltons are shown at the left. Proteins were detected with Coomassie blue stain (lanes a to d) or silver stain (lane e). GlbN, recombinant cyanoglobin; Or, origin.

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