cells and in vivo on various experimental tumor models (*21*, *24*). They appear to be a promising new class of antineoplastic agents. One of their possible mechanisms of action involves interaction with topoisomerase II–DNA complexes (*21*). The experimental data presented here suggest that these drugs might also promote intramolecular triplex formation in vivo (such as by stabilizing H-DNA structures) and therefore might interfere with transcription and replication, which represents a new potential mechanism of action for these antitumor drugs.

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29. Transcription assays were performed in a 100 mM NaCl, 10 mM $MgCl_2$ (pH 7.8), and 10 mM tris-HCl buffer, with 5 nM of a 267-bp Eco RI–Hph I fragment carrying the *bla* promoter from plasmid pBR322. *Escherichia coli* RNA polymerase [45% active molecules (*26*) prepared as described (*27*)] was added in a 120-μl transcription buffer and incubated for 45 min at 37°C in order to obtain a maximum of open complexes, which are unstable when the incubation is performed directly at a lower temperature (*26*, *28*). After preincubation, aliquots of 10 μl were withdrawn and distributed into 12 tubes that contained heparin at 150 μg/ml (final concentration) to remove all nonspecific complexes and to trap all free polymerases. After 1 min, the incubation was continued at 25°C. The oligonucleotide was added at increasing concentrations in the presence or absence of 1.5 μM BePI, and the mixture was incubated for 30 min. The four ribonucleotides were then added [adenosine 5′-triphosphate, guanosine 5′-triphosphate, and cytidine 5′-triphosphate at 500 μM and uridine 5′-triphosphate (UTP) at 10 μM including [α-^{32}P]UTP at 20 Ci/mmol], and the elongation was stopped after 10 min by the addition of 4 μl of a solution containing 50 mM EDTA, 2% SDS, and 20 μg tRNA. After two ethanol precipitations, the transcripts were heated for 2 min at 90°C and loaded on a 20% denaturing polyacrylamide gel.

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Hydrogen Exchange Measurement of the Free Energy of Structural and Allosteric Change in Hemoglobin

S. Walter Englander,* Joan J. Englander, Russell E. McKinnie, Gary K. Ackers, George J. Turner, Judy A. Westrick, Stanley J. Gill

The inability to localize and measure the free energy of protein structure and structure change severely limits protein structure-function investigations. The local unfolding model for protein hydrogen exchange quantitatively relates the free energy of local structural stability with the hydrogen exchange rate of concerted sets of structurally related protons. In tests with a number of modified hemoglobin forms, the loss in structural free energy obtained locally from hydrogen exchange results matches the loss in allosteric free energy measured globally by oxygen-binding and subunit dissociation experiments.

Much is now known about the structure of proteins but very little is known about how they work. In working proteins such as hemoglobin (Hb), structural changes convert the energy of ligand binding or other sources into a functionally useful form. It has not before been possible to localize and measure structural free energy and changes therein in any general way although this is fundamental for understanding structure-function relations. Results presented here indicate that measurement of the normally occurring hydrogen exchange behavior of proteins can provide this information.

The local unfolding model for protein hydrogen exchange (HX), diagrammed in Fig. 1, holds that slowly exchanging hydrogens are slow simply because they are blocked by hydrogen bonding and that exchange requires transient hydrogen bond breakage (*1*), which tends to occur in cooperative local unfolding reactions (*2*). Local unfolding and refolding occur constantly. Since the rate-limiting chemical exchange step is relatively slow, the kinetics of exchange of a set of peptide group NHs depends on the equilibrium constant of the unfolding reaction that exposes them to exchange (Eq. 1 in legend to Fig. 1). This connects HX rate with structural free energy [(*2*); see Eq. 2 in legend to Fig. 1].

From the unfolding model, some rather surprising predictions can be drawn. Hydrogens exposed to solvent by the unfolding of a cooperative structural unit should all tend to exchange at similar rates, independent of their relative exposure to solvent in the native state. However, chemical and physical mechanisms can operate to obscure this behavior (legend to Fig. 1). Deeper predictions of the unfolding model relate to change in the HX rate of hydrogens placed on a concerted local unfolding unit (unfoldon). When a modification alters the stability of a protein, this can be measured in a global, reversible denaturation experiment. The same modification will produce a change in the local unfolding equilibrium of any affected unfoldon and therefore a concerted change in the HX rate of all of the NHs governed by that unfoldon (Eq. 2b in legend to Fig. 1). Thus, measurement of a change in HX rate may localize and quantify a change in structural free energy that must otherwise be measured indirectly

S. W. Englander, J. J. Englander, R. E. McKinnie, The Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104–6059.
G. K. Ackers and G. J. Turner, Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, MO 63110.
J. A. Westrick and S. J. Gill, Department of Biochemistry, University of Colorado, Boulder, CO 80309.
*To whom correspondence should be addressed.

in terms of some global stability parameter.

In Fig. 2 are shown HX results for some allosterically sensitive peptide NHs in Hb, a set of five hydrogens placed near the NH_2-terminus of the α-chain and a set of four at the COOH-terminus of the β-chain (Fig. 3) (*3*, *4*). Selective measurement of these particular hydrogens was achieved by functional labeling (*3*–*5*) and fragment separation (*6*) methods. The hydrogens in each set exchange over a range of rates perhaps one order of magnitude wide, yet protein modifications cause all of the hydrogens in a set to move in unison to a new rate, faster by a common factor. The NHs in the α-chain set (Fig. 2A) exchange twice as fast in response to a specific chemical modification at the reactive sulfhydril, Cys93β (NES-Hb; see Table 1 and Fig. 3). In oxy Hb they all exchange nine times as fast. The NHs in the β-chain set (Fig. 2B) all move in unison to a new rate that is four times as fast when the phosphate allosteric effector is removed and eight times as fast with the NES modification. When oxygen is added, their rate increases 750-fold (190-fold without phosphate).

The HX rate changes due to specific chemical, mutational, and allosteric modifications were tested in this way. Modifications studied are listed in Table 1. Figure 3 diagrams some stabilizing, allosterically sensitive cross-links (*7*) that are removed, one or more at a time, by these manipulations. In each case the resulting destabilization caused a concerted increase in HX rate of the marker protons described, as in Fig. 2. This is the expected result for HX determined by a local unfolding reaction that transiently breaks the protecting hydrogen bonds and the stabilizing cross-links.

Changes in structural free energy due to these modifications were calculated from the measured HX rate changes according to Eq. 2b in the legend to Fig. 1. The results suggest that the two sets of allosterically sensitive NHs studied here sense the summed destabilization free energy of the allosteric structural transition of Hb. For example, in the deoxy to oxy structural transition, the ninefold acceleration in HX rate at each α-chain NH_2-terminus (Fig. 2A) indicates a destabilization of 1.2 kcal/mol at that position, or a total of 2.4 kcal/mol of Hb tetramer (Hb = $\alpha_2\beta_2$). The 190-fold acceleration at the β-chain COOH-terminus on oxygenation (the result when phosphate effector is absent) represents a destabilization of 2.85 kcal/mol at that locus, or 5.7 kcal/mol of Hb tetramer. Therefore, these two positions together sense a total of 8.1 kcal/mol in allosteric destabilization free energy (0°C, pH 7.4, without phosphate effector). This compares with the estimate of 8.2 to 8.3 kcal/mol of tetramer obtained from O_2-binding experiments (at 5°C) and subunit dissociation experiments [measured at 21.5°C, calculated from the experimental enthalpies (*8*)].

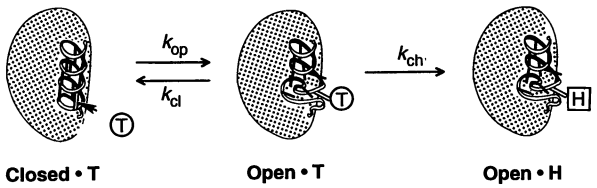

Fig. 1. Diagrammatic representation of a local unfolding reaction. A section of helix is pictured to unfold transiently and reversibly, breaking a set of contiguous hydrogen bonds. Protein NH (pictured with tritium label) can exchange with solvent hydrogens only from the open state, the occupation of which is determined by the unfolding (opening) equilibrium constant, K_{op}. The HX rate determined by this sequence can generally be represented as in Eq. 1.

$$k_{ex} = k_{op}k_{ch}[OH^-]/(k_{op} + k_{cl} + k_{ch}[OH^-]) \approx K_{op}k_{ch}[OH^-] \quad (1)$$

Here k_{op} and k_{cl} are opening and closing rate constants and $k_{ch}[OH^-]$ is the chemical exchange rate characteristic of the freely exposed hydrogen (specific base–catalyzed above pH 3). In a real case, the NHs in an unfolding reaction may not all exchange at the same rate. Some dispersion in rates is imposed by chemical inductive effects of nearest neighbor side chains (*15*). Physical effects may also intervene, such as partial protection of some residues in the open state. Equation 2a (where R is the gas constant and T is temperature) recognizes that K_{op} is determined by the equilibrium free energy of the opening reaction ($\Delta G°_{op}$).

$$\Delta G°_{op} = -RT \ln K_{op} \quad (2a)$$

$$\delta\Delta G°_{op} = -RT\delta \ln K_{op} = -RT \ln (k_{ex,1}/k_{ex,2}) \quad (2b)$$

A change in the free energy of unfolding ($\delta\Delta G°_{op}$) will produce a multiplicative change in K_{op} and therefore a multiplicative change in the HX rate of all the hydrogens in the set, as in Eq. 2b. Equation 2b assumes that the change in structure or interaction represented by $\delta\Delta G°_{op}$ affects the native state only and not the energy level of the transiently unfolded state.

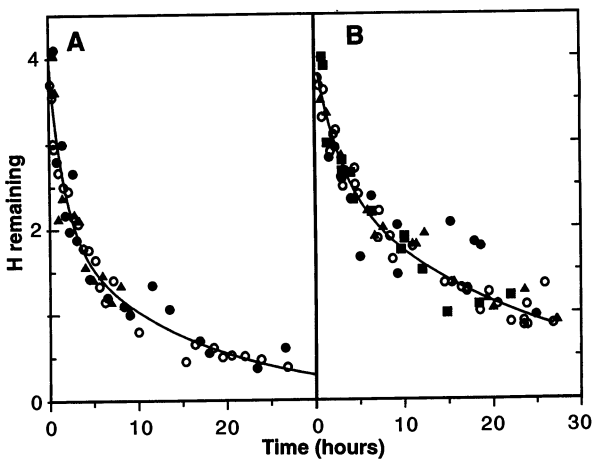

Fig. 2. Hydrogen-tritium exchange curves for allosterically sensitive peptide NH at (**A**) the α-chain NH_2-terminus and (**B**) the β-chain COOH-terminus of human Hb (pH 7.4, 0°C, 0.1 M NaCl, 0.1 M phosphate, ±20 mM pyrophosphate, and 5 mM ferrous ammonium sulfate; Hb concentration ~0.5 mM in tetramers). HX data are shown for deoxy Hb and several modified forms. For each modified Hb, the HX time scale is shown multiplied by a factor that brings its exchange curve into consonance with the unmodified deoxy Hb curve (two-exponential fit). (A): (○), HbA, ×1; (▲), NES, ×2; (●), oxy Hb, ×9. (B): (○), HbA, ×1; (■), no PP_i, ×4; (▲), NES, ×8; (●), oxy Hb, ×750. The multiplying factor was computer-determined. In these experiments, allosterically sensitive sites were selectively tritium-labeled by the functional labeling method (*3*–*5*). Hemoglobin was initially exchanged-in in the fast-exchanging, oxy form in tritiated water (THO) for 35 min. Both allosterically sensitive and insensitive NH sites that exchange in 35 min become labeled. Hemoglobin was then deoxygenated (sodium dithionite), and exchange-out was immediately initiated by removing free $^3H^1HO$ (short gel filtration passage through a 1 cm by 6 cm column of G25 fine Sephadex). At allosterically insensitive sites tritium label is soon lost, because exchange-out proceeds at the same rate as did exchange-in. At allosterically sensitive sites, now in their slow form, tritium is locked into a more slowly exchanging form. Thus, after a short exchange-out time the hemoglobin sample selectively retains label essentially at allosterically sensitive sites. Tritium label remaining unexchanged at various protein locations as a function of exchange-out time was measured by a fragment separation method (*6*). Hemoglobin samples prepared as just described were taken after various exchange-out times, placed into slow HX conditions (pH 3, 0°C) by passage through a 3-cm Sephadex column (<1 min), and proteolyzed with pepsin (5 min). The fragments were separated by high-pressure liquid chromatography (~20 min), and the label on each fragment was counted by liquid scintillation. The allosterically sensitive NHs at the α-chain NH_2-terminus were measured on the fragment α1–29 (only four of the five sensitive NHs in the set are labeled in the 35-min exchange-in). The four sensitive NHs at the β-chain COOH-terminus (Fig. 3) were recovered and measured on the fragment β130–146. A correction was made for the calibrated loss of label during the fragment separation analysis (19 and 33%, respectively, for the α- and β-chain sets). A low-level background curve due to labeled allosterically insensitive sites was generated and subtracted as described (*4*).

For each modification, Table 1 compares the loss in structural free energy calculated from HX measurements with the loss in allosteric free energy measured at a global level in subunit dissociation and O_2-binding experiments. Good agreement is found.

A paradox arises. If the total cooperative free energy in Hb and modified Hbs can be measured at the α-chain NH_2-terminus and the β-chain COOH-terminus taken together, how is it that all or part of the cooperative energy can also be sensed at other positions, for example, in the β-chain FG segment by HX measurements (*3*) and, of course, at the heme binding sites in O_2-binding experiments? A general explanation is that the free energy of a given linkage can appear in multiple, independent measurements. If an individual cross-link is severed, such as the intrachain salt link connecting His146β at the β-chain COOH-terminus and Asp94β in the FG segment (Fig. 3), separate measurements of destabilization energy at the COOH-terminal and FG segments (for example, by HX) will both reflect the loss of the same stabilizing cross-link. The total free energy of the cross-link will appear also in any functionally linked equilibrium (*9*, *10*) in which the linkage is severed, and the bond free energy can be measured thereby, as in Table 1. A more specific explanation for the special role of the two terminal positions reported on here must be sought in the detailed construction of the allosteric machinery of Hb.

Most of the exchangeable hydrogens in Hb do not respond to the allosteric transition (*11*). However, our results show that the loss of a single stabilizing bond or multiple bonds causes some specific sets of peptide group NHs to move in concert to a new HX rate, faster by factors ranging from 2 to 750. For a number of modifications, structural free energy changes calculated according to the local unfolding model match allosteric free energy changes measured externally by established methods. No such relation is suggested by any other known model for protein HX. Cooperative sets of exchanging NH have also been seen in other proteins (*12*), suggesting that the local unfolding mechanism occurs widely.

The results summarized in Table 1 used known allosterically sensitive hydrogens to identify and quantitatively evaluate some interactions that participate in the regulatory function of Hb. These results show how HX measurements can help to delineate, in terms of definable bonds and their measurable energies and interactions, the network of interactions that Hb or other proteins use to produce their various functions.

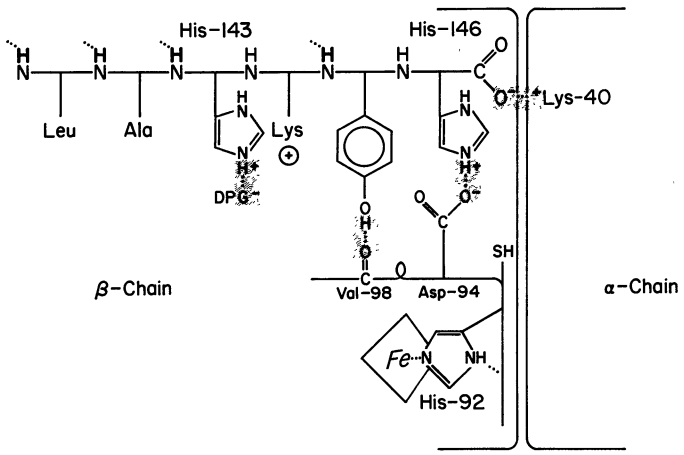

Fig. 3. Interactions in the vicinity of the allosterically sensitive peptide NHs at the β-chain COOH-terminus (*7*). The β-chain heme is shown coordinated to the proximal histidine (His-92) on the F-FG segment. This segment is joined to the COOH-terminal segment by a number of allosterically sensitive bonds, indicated by shading. Each modification tested breaks one or more of these cross-links, as indicated in Table 1. The hydrogen-bonded NHs believed to account for the HX behavior measured at the β-chain COOH-terminus (Fig. 2B) are indicated by dotted lines.

Table 1. Loss of cooperative free energy in Hb measured by hydrogen exchange and global methods. Free energy values, in kilocalories per mole of tetrameric Hb molecules, represent the loss in structural stability (HX) or in allosteric cooperativity [subunit dissociation and O_2 binding (*13*)] caused by each modification listed (all at pH 7.4). The change in local stabilization free energy relative to deoxy Hb was calculated from HX results at the α-chain NH_2-terminus and the β-chain COOH-terminus with Eq. 2b (see legend to Fig. 1); the sum of these two values is listed for each modified Hb form. Entries under "subunit dissociation" and "O_2 binding" were calculated with Eqs. 5 and 6, respectively (*13*). The Hb modifications are as follows: No PP_i, deoxy Hb in bis-tris buffer with no phosphate effector, such as 2,3-diphosphoglycerate (DPG) or pyrophosphate (PP_i); Bunbury, Asp94β → Asn; NES, *N*-ethyl succinimydyl derivative of Cys93β; desHis, His146β removed with carboxypeptidase A; Kariya, Lys40α → Glu; and NES-desArg, the NES modification together with excision of Arg141α (carboxypeptidase B treatment), measured in the presence of PP_i, an analog of DPG.

Modification	Hydrogen exchange	Subunit dissociation		O_2 binding
		$(\delta^0\Delta G_{diss})$	$(\delta^0\Delta G_{diss} - \delta^4\Delta G_{diss})$	
No PP_i	0.75			0.4
Bunbury	1.1	0.5	0.2	0.6
NES	3.0	3.0	3.6	2.5
DesHis	3.0	2.7	2.7	3.9
Kariya	4.5	5.7	5.7	6.3
NES-desArg	6.2			6.9
Oxygenation	8.1	8.2	8.3	8.3

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13. Cooperative free energies obtained from O_2-binding measurements and subunit dissociation experiments can be related by a thermodynamic linkage diagram (Eq. 3):

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

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 ΔG values are defined in Eq. 3 from left species to right and from top species to bottom. The cooperative free energy, ΔG_{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.

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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

Patricia N. Fultz,* Peter Nara, Francoise Barre-Sinoussi, Agnes Chaput, Michael L. Greenberg, Elizabeth Muchmore, Marie-Paule Kieny, Marc Girard

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-

P. N. Fultz, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294.
P. Nara, National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD 21701.
F. Barre-Sinoussi, A. Chaput, M. Girard, Institut Pasteur, 75015 Paris, France.
M. L. Greenberg, Duke University Medical Center, Durham, NC 27710.
E. Muchmore, Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center, New York, NY 10016.
M.-P. Kieny, Transgene, 67082 Strasbourg, France.
*To whom correspondence should be addressed.