

# Human Immunodeficiency Virus (HIV) Tat-reactive Antibodies Present in Normal HIV-negative Sera and Depleted in HIV-positive Sera. Identification of the Epitope

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## Summary

We have detected, in sera of normal human immunodeficiency virus (HIV)-free subjects, IgM antibodies reactive with the Tat protein of HIV in significant titers and at very high frequency, and, in HIV-positive sera, progressively lower titers as HIV pathogenesis ensues. Epitope analysis indicates that the Tat-reactive antibodies of both HIV-negative and HIV-positive sera are homologous, suggesting, therefore, that their decline in HIV-positive sera may represent attrition of a host defense factor. The identified epitope displays minimal homology with that previously defined for another set of IgM antibodies shown to be present in normal sera, deficient in HIV-positive sera, and postulated to be natural antibodies. We propose that the Tat-reactive antibodies, as well, are a set of natural antibodies and that the normal humoral immune system includes a repertoire of antibodies, nonimmunogenic in origin, that contribute to immune homeostasis and, consequently, to host resistance to HIV pathogenesis.

The progression of HIV pathogenesis from infection to AIDS is highly variable in rate and in pathophysiological manifestations. While some variability is attributable to genetic variance of the virus, there is great likelihood that host factors, some possibly not yet identified, are significant determinants of, for example, duration of latency between infection and morbidity. We have directed our attention to one arm of the humoral immune system in which such factors might reside: the natural antibody repertoire.

Among the various functions or *raison d'être* proposed for natural antibodies is that they may constitute a "first line of defense" against invading infectious agents (1). In consideration of that putative function of natural antibodies as a mechanism of host defense against HIV, we have previously provided evidence that certain subsets of natural antibodies are present in sera of healthy HIV-negative subjects and deficient or absent in sera of patients diagnosed with AIDS (2, 3). In the more recent study (3) the antigenic specificity of the subset of natural antibodies was defined, and decline of those antibodies in HIV-positive sera was shown to be related to the imminence of AIDS. The antigens with which the subsets of natural antibodies were detected were those derived from sperm surface components (2, 4) and from protamine, a sperm-unique nuclear protein (3, 5). In this study we have identified what appears to be another set of natural antibodies indigenous to normal sera, depleted in the course of HIV infection and,

of particular significance, specifically reactive with Tat, a regulatory transactivation protein of HIV (6). Tat has been shown *in vitro* to activate viral replication (7, 8), to be exocytosed (9), and to adhere to various classes of cells (10). If those do indeed reflect events *in vivo*, the presence, in the early postinfection period, of Tat-reactive antibodies in the plasma may provide a mode of inhibition of Tat activity, impedance of stimulation of HIV replication, and, consequently, maintenance of latency or asymptomatic status.

## Materials and Methods

**Sera.** HIV-positive sera were collected from blood specimens of individuals at risk for AIDS during the period 1983–1990, and stored in small aliquots at  $-60^{\circ}\text{C}$ . Diagnosis at specimen collection and at subsequent clinical examinations allowed the sera to be assigned to three classes: (a) AIDS, (b) AIDS within 1 yr, and (c) asymptomatic or state of latency for  $>1$  yr. The HIV-negative sera were selected randomly from a store of 200 specimens, including donations from laboratory personnel and discards from clinical laboratories identified by age, sex, and "no clinical findings." Those specimens also were collected throughout the period 1983–1990, and stored at  $-60^{\circ}\text{C}$ . HIV positivity for all sera was determined by immunotransfer as described (3) or by report from certified clinical laboratories.

**Antigens.** Recombinant HIV Tat protein (complete, residues 1–86) representing the BH10, HxP2 isolates (11, 12) and HIV Nef

expressed in *Escherichia coli*, HIV gp 120, and HIV p24 expressed in baculovirus, were obtained from American Biotechnologies Inc. (Cambridge, MA). Synthetic truncated Tat was a gift, prepared as described (13). 11 overlapping dodecapeptides of Tat, including residues 1–82, constructed from the sequence described (14), were prepared by Multiple Peptide Systems (San Diego, CA). pp, a dodecapeptide representing residues 33–44 of human protamine 2b (15, 16) was prepared by The Rockefeller University Protein Sequencing Service. All peptides were synthesized by the method of Merrifield (17), and amino acid content of each was verified by the Mass Spectrometric Biotechnology Resource of The Rockefeller University.

**ELISA.** IgM reactivity of each serum with Tat, peptides of Tat, gp120, p24, and Nef was determined by ELISA as described (3, 5, 18). Briefly, 50  $\mu$ l of 10  $\mu$ g antigen/ml PBS was placed in each well (96-well microtiter plates; Dynatech Laboratories, Inc., Alexandria, VA), incubated at room temperature for 3 h, blocked with 3% BSA overnight at 5°C, then with 1% preimmune rabbit serum for 1 h (blocking with preimmune rabbit serum eliminated background attributable to the second antibody). 50  $\mu$ l of the test serum (1:100) was added to the wells, incubated for 2 h, followed by 50  $\mu$ l of the peroxidase-labeled second antibody: IgG isolated from serum of a rabbit immunized with purified total IgM from pooled normal sera, for 1.5 h. 50  $\mu$ l substrate, orthophenylene diamine (0.2 mg/ml) was added to each well, allowed to incubate for 30 min at room temperature, and the reaction stopped by the addition of 50  $\mu$ l 2.5 N H<sub>2</sub>SO<sub>4</sub>. After removal of each reagent, the wells were washed 20 times with 0.05% Tween 20/PBS. The OD at 490 nm, of each well, was read in a microplate reader (MR 700; Dynatech Laboratories, Inc.). All appropriate controls were included and caveats for correction for background and other methodologic sources of error in the ELISA were observed (19). Each plate included both HIV-positive and HIV-negative sera, each reaction was carried out in duplicate, and each serum was assayed two to five times. Throughout the study, a single normal serum was assayed for reactivity with HIV Tat 48 times with a mean corrected OD value of 0.50 for 1:100 dilution of serum with 10  $\mu$ g/ml of Tat (SEM = 1.2). ELISA with pp as antigen and calculation of the proportionate titer of the low affinity subset of pp-reactive IgM, designated as the natural antibody subset, was carried out as described (3).

**Dithiothreitol (DTT) Treatment of Peptides.** To determine whether the epitope of Tat with which the serum IgM antibodies are reactive is represented by the linear sequence of Tat peptide no. 4 or by a conformation-dependent structure resulting from S-S bonding

of the cysteinyl residues, assay of the reactivity of HIV-negative and HIV-positive sera with Tat peptide no. 4 was carried out by the conventional procedure with the peptide in PBS and, in parallel, with Tat peptide no. 4 in 10 mM DTT/PBS. The same procedure was carried out for serum IgM reactivity with pp (see Table 2).

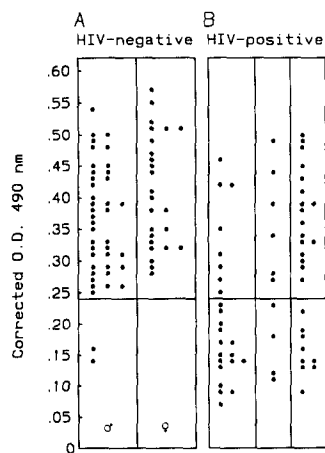
**Immunotransfers of IgM and IgG Reactivity.** Immunotransfers of representative sera with each of the HIV proteins was carried out as follows. A wide PAGE of each HIV protein (gp120, p24, Nef, Tat, synthetic truncated Tat) was prepared and transferred to Immobilon P membrane. A pair of 2-mm strips, representing 1.5  $\mu$ g protein each, were incubated in 1:100 dilution of serum for 2 h at room temperature. The strips were washed with PBS/Tween 20, then one of each pair was incubated in peroxidase-labeled rabbit IgG anti-human IgM and the other in anti-human IgG, washed, and incubated in substrate, 3-amino-9-ethyl carbazole, for 1 h. Reaction was stopped by washing (H<sub>2</sub>O) and air drying.

## Results

**Assay of Sera for IgM Reactivity with Tat.** Sera of 66 HIV-negative and 60 HIV-positive adult males and females, none of whom had received antiviral or immune-corrective therapy, were assayed for IgM antibodies reactive with HIV Tat. As shown (Fig. 1 A), of the HIV-negative sera from clinically normal subjects, 100% of the female and 95% of the male sera had titers within a circumscribed range. For the HIV-positive sera (Fig. 1 B), 66% of those diagnosed with AIDS (class 1), 40% of those from patients for whom a diagnosis of AIDS was entered within 1 yr (class 2), and 35% of those who remained AIDS free for >1 yr (class 3) had titers below that range. These data suggest that progression to AIDS is accompanied by a decline in HIV-positive sera of a constant component of normal sera: a set of IgM antibodies reactive with HIV Tat protein.

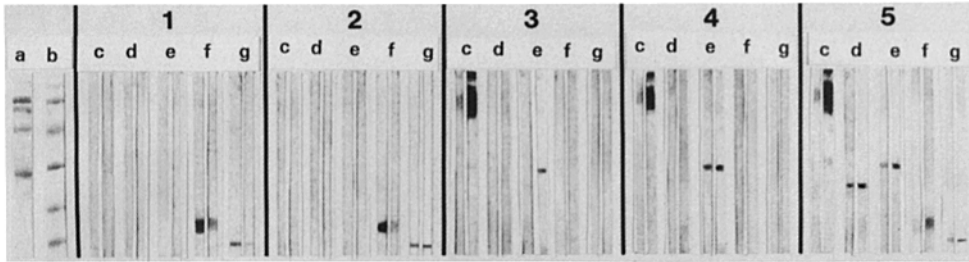
**HIV-negative Sera Are Not Reactive with HIV Proteins Other than Tat.** Further creditation for the specificity and unique occurrence of the Tat-reactive antibodies (Fig. 1) was sought by inspecting the reactivity of a representative group of sera with other HIV proteins as well as Tat, and by utilizing another method of immunochemical analysis (Fig. 2). The HIV proteins selected for this comparison were the structural proteins gp120 and p24 and, in addition to Tat, another regulatory protein, Nef. The most frequently used criterion for HIV positivity is that of reactivity with the structural proteins. There is well-accepted evidence that antibodies to the envelope protein gp120 appear soon after infection and are present at all subsequent stages (20), while antibodies to the gag protein, p24, decline as disease progresses (21). The role of the Nef protein in HIV replication or expression in vitro has not been clearly defined (22). Detection of antibodies to HIV Nef in sera of patients at all stages of HIV infection has been reported (for review, see reference 23); that prevalence, however, has been questioned (for review, see reference 24).

Detection of Tat-reactive IgG antibodies in some HIV-positive sera has been reported and interpreted as evidence of immune response to the viral protein; no reactivity with Tat by IgG of HIV-negative sera was observed (25, 26). Our own interest has centered on IgM antibodies as the principle class identified as natural antibodies. However, to correlate

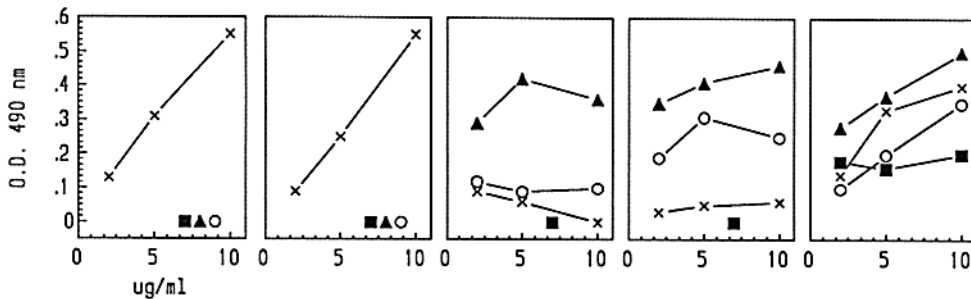


**Figure 1.** Titers, measured by ELISA and expressed as OD of IgM antibodies reactive with HIV Tat in sera of: (A) clinically normal, HIV-negative males ( $n = 41$ ) and females ( $n = 25$ ); (B) HIV-positive (1) diagnosed with AIDS at specimen collection ( $n = 24$ ); (2) diagnosed with AIDS within 1 yr from specimen collection ( $n = 10$ ); (3) asymptomatic or state of latency for >1 yr ( $n = 26$ ).

A



B



**Figure 2.** (A) Immunotransfers of IgM and IgG reactivity of representative sera from the classes described in Fig. 1, with HIV structural proteins gp120, p24, the regulatory protein Nef, the recombinant Tat, and a synthetic truncated Tat. (Lane a) Molecular mass markers on 10% polyacrylamide gel: 200, 130, 92, 66, and 43 kD; (lane b) molecular mass markers on 12% gel: 92, 66, 43, 30, 20, and 14.4 kD. For each of the following lanes, the left hand member of the pair is IgM and the right hand member is IgG: (lane c) gp120, 10% gel; (lanes d-g) 12% gel; (lane d) p24; (lane e) Nef; (lane f) recombinant Tat; (lane g) synthetic Tat (residues 1-68). Sera nos. 1 and 2 are HIV negative; sera nos. 3 and 4 are from class 1, AIDS; serum no. 5 is from class 3, state of latency. The strong band at ~240 kD on the IgG member of lane c, sera nos. 3, 4, and 5, and the weak band at 45 kD for sera 3 and 5 were proven, by reactivity with a mAb

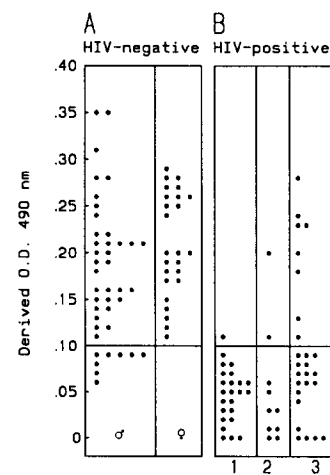
to gp120, to be, respectively, a gp120-derived dimer and peptide (not shown). (B) ELISA of IgM reactivity of the same sera presented in A, paralleling and supporting the inferences of the data for the reactions displayed in A. 1:100 dilution of each serum was assayed for IgM reactivity with 2, 5, and 10  $\mu$ g/ml of each protein, and the corrected OD values were plotted (18). x, Tat; ▲, gp120; ■, p24; ○, Nef.

the data of this report with those previously published (25, 26), immunotransfers of IgG reactivity of each serum with each HIV protein were included (Fig. 2 A). As shown (Fig. 2 A), the IgG reactivity of each serum with each of that panel of HIV proteins is in accord with the referred published data.

Sera nos. 1 and 2 (HIV negative) show no reactivity with gp120; sera nos. 3, 4, and 5 (HIV positive) show weak IgM and strong IgG reactivity with gp120. Only serum no. 5 (state of latency) displays reactivity, IgM and IgG, with p24. The three HIV-positive sera (nos. 3, 4, and 5) show IgG reactivity with Nef, and sera nos. 4 and 5 show IgM reactivity, as well. In accordance with the data of Fig. 1, the HIV-negative sera (nos. 1 and 2) show high IgM reactivity with Tat, while the two AIDS sera (nos. 3 and 4) show no discernible reactivity, and the HIV-positive, state-of-latency serum show moderate IgM reactivity with Tat. Each display of IgM reactivity with Tat is accompanied by display of IgG reactivity, although the relative densities of the IgM and IgG bands differ for the HIV-negative and HIV-positive sera. Reactivity with the truncated synthetic Tat (Fig. 2, lane g) is comparable, for each serum, with that with the recombinant Tat (lane f), thus assuring that the observed reactivity is not attributable to autogenous proteins of the bacterial vector. The IgM reactivities displayed for each serum by the ELISA (Fig. 2 B) are markedly consistent with those detected by the immunotransfers (Fig. 2 A).

*Assay of Sera for Natural IgM Antibodies Reactive with pp.* This probe for natural antibodies reactive with HIV Tat was suggested by the linear sequence of the epitope for the set of protamine-reactive IgM antibodies previously character-

ized (3, 5). That epitope was identified as one with a concentration of four arginyl residues, consisting of a triplet plus one within a six-residue sequence. Therefore, the identification of an arginine-rich sequence in HIV Tat (27) was provocative and suggested that the same set of natural antibodies might display reactivity with Tat. In that regard, each of the sera assayed for Tat-reactive IgM antibodies (Fig. 1) was assayed



**Figure 3.** Titers of the same sera as those of Fig. 1, measured by ELISA and expressed as the derived OD representing the subset of low affinity IgM antibodies, designated as natural antibodies (3), reactive with pp, a peptide (residues 33-44) of human protamine 2b (5). The ELISA protocol and formula for deriving the proportional titer of protamine-reactive IgM representing the low affinity subset and designated as natural antibodies, distinct from a subset of high affinity IgM antibodies attributable to immunoinduction by protamine, have been described (3). (A) With the lower limit of the normal range of titers designated as .10, 11% of the HIV-

negative sera show titers below that limit. (B) Of the HIV-positive sera, 96% of class 1 (AIDS), 80% of class 2 (AIDS within 1 yr), and 71% of class 3 (state of latency) show titers below the designated lower limit of normal range.

also for titer of natural IgM antibodies reactive with a peptide, pp (Fig. 3), representing a 12-residue sequence of protamine and including one copy of the deduced epitope (5).

The protocol and formula for deriving the proportionate titer of protamine-reactive IgM antibodies comprising the low affinity subset and designated as natural antibodies, distinct from a subset of high affinity IgM antibodies attributable to immunoinduction by protamine of sperm, have been described (3). By application of that formula, the titer of natural antibodies reactive with pp was derived for each serum (Fig. 3). As shown, the general distributions of pp-reactive (Fig. 3) and Tat-reactive (Fig. 1) natural IgM antibodies in the various groups of sera are similar. With the lower limit of the normal range designated as 0.10, only 11% of the HIV-negative sera show titers of reactivity with pp below that limit (Fig. 3 A), while, of the HIV-positive sera (Fig. 3 B), 96% of those from patients diagnosed with AIDS (class 1), 80% of those from patients diagnosed with AIDS within 1 yr (class 2), and 71% from those who remained AIDS-free for >1 yr (class 3) show titers below the normal range.

*Probe for the Epitope of Tat Recognized by Serum IgM.* Identification of the epitope was sought by assay of sera of each of the two groups, HIV negative and HIV positive, for IgM reactivity against a series of overlapping peptides of Tat, pp, and the total Tat (Table 1). The apparent homology of

arginine distribution in pp and Tat peptide no. 8 engendered the expectation that both would be recognized by the same set of IgM antibodies. It was particularly surprising, therefore, to observe no reactivity by any of the sera with the arginine-rich Tat peptide no. 8, nor with any of the Tat peptides other than peptide no. 4 (Table 1). All sera, of both groups, were reactive to varying degree with Tat peptide no. 4, with pp, and with total Tat. The limited homology between the two dodecapeptides, Tat peptide no. 4 and pp, suggests that this study has revealed two sets of natural antibodies: one reactive with an epitope characteristic of Tat peptide no. 4, and another with an epitope indigenous to pp, but not present in Tat peptide no. 4 nor in any linear sequence of Tat.

The assays of serum IgM reactivity with Tat and with the peptides of Tat displayed in Table 1 were carried out in the conventional protocol, which included solution of Ag in PBS. The presence of five cysteinyl residues in Tat peptide no. 4 suggested that the epitope might be dependent upon S-S bond-induced conformation. Therefore, HIV-negative and HIV-positive sera of each class were assayed for reactivity with Tat peptide no. 4 in 10 mM DTT and compared with the reactivity of peptide no. 4 in conventional (nonreducing) medium (Table 2). The sera were assayed also for comparative reactivity of pp in conventional and reducing solution.

**Table 1.** Epitope Specificity for HIV Tat-reactive IgM Antibodies

No.	Residues	Tat peptides	OD 490 nm					
			HIV-neg			HIV-pos		
			Serum: 1	2	3	4	5	6
1	1-12	Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys	-	-	-	-	-	-
2	8-19	Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys	-	-	-	-	-	-
3	15-26	Gly Ser Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr	-	-	-	-	-	-
4	22-33	Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His	0.26	0.31	0.13	0.11	0.21	0.21
5	29-40	Lys Cys Cys Phe His Cys Gln Val Cys Phe Ile Thr	-	-	-	-	-	-
6	36-47	Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr	-	-	-	-	-	-
7	43-54	Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg Arg Gln	-	-	-	-	-	-
8	50-61	Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly	-	-	-	-	-	-
9	57-68	Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser	-	-	-	-	-	-
10	64-75	Thr His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser	-	-	-	-	-	-
11	71-82	Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr	-	-	-	-	-	-
pp		Ser Cys Arg His Arg Arg Arg His Arg Arg Gly Cys	0.24	0.39	0.22	0.26	0.18	0.20
Tat (total)	1-86		0.35	0.49	0.33	0.09	0.17	0.20

HIV-negative sera (nos. 1-3) and HIV-positive sera (nos. 4-6) were assayed for IgM reactivity with a series of overlapping 12 residue peptides of Tat, pp, a 12-residue peptide of protamine (5), and with the total recombinant Tat protein. Sera nos. 4 and 5 were class 1, AIDS and serum 6 was class 3, state of latency (Fig. 1 B). 50 µl of 1:100 dilution of serum was tested against 50 µl of 10 µg/ml of each peptide in accordance with the ELISA protocol, and the OD for each serum/peptide reactivity was corrected for background (18). Reactivity by all sera was displayed with Tat protein, with pp, and with Tat peptide 4, but with no other Tat peptide. Despite the apparent homology of arginine distribution in Tat peptide 8 and pp, no reactivity with peptide 8 was displayed by any of the sera tested. Levels of reactivity with pp were consistent with those previously determined for protamine and other protamine-derived peptides (3) and with the data of Fig. 3. The reactivity of each serum with the complete Tat protein was in accordance with the data of Fig. 1.

**Table 2.** Decline in Serum IgM Reactivity with Tat Peptide No. 4 and pp, after DTT Treatment of Peptides

Sera	Antigen	n	Percent decline (M)
HIV-neg	Tat pep. no. 4	18	92.6
HIV-pos.	Tat pep. no. 4	17	93.5
HIV-neg.	pp	18	25.8
HIV-pos.	pp	14	28.5

The data of Table 2 show that the reactivity of the sera with Tat peptide no. 4 is virtually eliminated when the antigen is in the reduced state, thus establishing that the epitope on Tat peptide no. 4 is conformation dependent. With regard to pp, the presence of DTT resulted in only moderate decline in reactivity, suggesting that the epitope of pp recognized by serum IgM has little dependence upon the conformation induced by S-S bond between the two cysteinyl residues, and confirming the previous observations that the repetitive epitope of protamine is that of a triplet plus one of arginine in a linear six-residue piece (5). The demonstration of the difference in response to DTT treatment by the two peptides, Tat no. 4 and pp, serves, fortuitously, to validate the discriminatory capability of that treatment for detecting S-S bond-dependent conformational epitopes and supports the interpretation that the epitope on HIV Tat is a conformational one that is recognized by both HIV-positive and HIV-negative sera.

## Discussion

The primary observation reported here is provocative: all sera of a randomly selected group of clinically normal, HIV-negative individuals contain a measurable titer of IgM antibodies reactive with the protein product of the HIV *tat* gene. Within presently accepted concepts of immunology, those antibodies are attributable to: (a) immunologic induction by an exceedingly ubiquitous exogenous antigen homologous with the region of Tat with which those antibodies are reactive; or (b) nonimmunogenic origin, therefore natural antibodies. Since the observations reported here also suggest that depletion of those antibodies occurs concomitantly with the progression, after HIV infection, to AIDS, we have accorded greater credibility to the latter.

Among the characteristics attributed to natural antibodies since their existence was postulated by Jerne in 1955 (28) are: preimmune origin or nonimmunogenic induction (1, 3, 5) with adaptability to antigenic demand (29, 30), capability of binding to autogenous or exogenous antigens (31), poly-

or monoreactivity (30, 31), IgM of low binding affinity (3, 32, 33) secretion by CD5 B cells (1, 33-36), participation in idiotypic networks with a role in maintenance of homeostasis (37-43), association with autoimmunity (44, 45), and first line of defense against invading infectious agents (1). The latter, in its most simplistic interpretation, presents a framework into which the data of this report may fit. That interpretation would allow that the Tat-reactive antibodies are a component of the neonatal or preimmune repertoire, are retained or replenished at significant titer throughout life unless the B cell lines of their origin are demolished (by disease, autoimmunity?), and, upon encounter with a foreign antigen that they recognize, establish an attack similar to that of neutralizing antibodies, maintain that attack for a host-variable limited period, then decline.

On the other hand, it should be noted that the distribution of titers of Tat-reactive IgM antibodies in the various classes of HIV-positive sera allows an alternative to that interpretation: in the postinfection pre-AIDS period (class 3), an immune response to Tat may occur, resulting in a set of induced Tat-reactive antibodies, possibly unrelated to the natural Tat-reactive antibodies. However, the implication of the data of Table 2, that the epitope for the Tat-reactive antibodies of HIV-positive sera is identical with or closely related to that of the HIV-negative sera, while not precluding that possibility, does not strongly support it. Another possible consideration, and one that is consistent with the epitope study (Tables 1 and 2), is that the presence of the Tat-reactive antibodies in HIV-positive sera might be consonant with the hypothesis that the immune response to HIV is under idiotypic network control (46-48) with the natural anti-Tat antibodies functioning as Ab1. The possibility that the natural anti-protamine antibodies, represented in this study as the pp-reactive set, may, in homosexual practice, be a factor in perturbation of immunologic equilibria such as idiotypic networks has been discussed in the context of the molecular, cytologic, and ontogenetic characteristics of protamine, a sperm-unique nuclear protein that is synthesized *de novo* in the testes of the postpuberal male. (2, 3).

Thus, whatever the nature of the subsequent humoral response, the data of this report suggest that the host's innate store of natural antibodies may provide the first retardation of the progression of HIV pathogenesis. Might that store be replenished? Could that replenishment be a basis for the slower progression to AIDS in HIV-infected hemophiliacs (49), for improvement in immune status of AIDS patients who have received plasma from "healthy HIV-infected individuals" (50), or for the HIV-infected pediatric patients who have received intravenous immunoglobulins obtained from the "blood of normal, healthy donors" (51) and have benefitted (52)?

We thank Drs. V. G. Allfrey, J. Laurence, and S. Astrin for reading the manuscript and for many helpful discussions, and J. Laurence, J. L. Brown, and J. S. Rodman for sera and relevant clinical data.

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Received for publication 16 December 1991 and in revised form 6 February 1992.

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