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Antigen HIV Markers for Clinical Manifestation and Prevention of HTLV-III/LAV Infections

Annual Report

Tun-Hou Lee

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The major objective of the proposed study is to identify HIV gene products and to determine if antibodies elicited by these products can be used to predict clinical outcome of HIV infection. During the first year, studies were conducted to map antigenic domains of gp120, and to investigate if HIV contains as yet undiscovered genes in its genome. Analysis of serum reactivity to various gp120 peptides revealed that antibodies to carboxyl portion of gp120 were more frequently detected in infected individuals who were at earlier stages of HIV infection or in those who were less likely to progress to AIDS. A new HIV gene located in the central region of the genome was also identified. This newly identified gene encodes a 16 kd protein. Antibody to this 16 kd protein was detected in HIV seropositive individuals. Unlike antibody to other HIV proteins, the prevalence of antibody to the 16 kd protein is elevated in patients with AIDS. Because no analogous coding region has been identified in HIV-2, the antibody to the 16 kd protein may serve as a marker to distinguish HIV-1 infection from HIV-2.

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FOREWORD

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

Table of Contents **Pages**

Section 1. Association Between Antibody to Envelope Glycoprotein gp120 and the Outcome of HIV Infection

Without a thorough understanding of the natural immune response produced during Human Immunodeficiency Virus (HIV) infection, most of our current approaches toward the development of envelope-based vaccines for HIV infection are fundamentally empirical in nature. It is conceivable that through empirical excercises an effective vaccine for the Acquired Immunodeficiency Syndrome (AIDS) can eventually be developed. One alternative to the trial and error approach is to design a vaccine based on the understanding of the role of natural immunity in disease pathogenesis.

Infection by HIV has been known to elicit strong antibody response to the envelope glycoprotein, gp120. One unanswered question relevant to the vaccine development is whether HIV infected individuals benefit from naturally produced gp120 antibodies. At present, there are two opposing views. One suggests that naturally produced gp120 antibodies play no significant role in preventing disease progression since gp120 antibodies are readily found in AIDS patients. The other suggests that infected individuals may benefit from naturally produced gp120 antibodies. Two observations support this latter view. First, no genomically divergent strains of HIV have been isolated from seropositive individuals known to have multiple sex partners (1). Second, broadly reactive neutralizing antibodies directed to gp120 have been detected in seropositive individuals (2). The former observation is compatible with the notion that protective immunity generated from primary HIV infection may be effective in preventing secondary exposure (3). The latter observation suggests that naturally produced gp120 antibodies may also be able to neutralize virus in vivo, which may help to slow down the course of disease progression (4).

Results presented here are summaries of several seroepidemiological studies.

We compared gp120 antibody response of HIV-infected individuals who had different clinical manifestations to determine if naturally produced gp120 antibodies are correlated with the outcome of HIV infection.

Two Major Types of gp120-binding Antibodies

Operationally speaking, there are two types of gp120-binding antibodies. One, designated gp120(NR), is directed to antigenic epitopes which require the presence of disulfide bonds to maintain its conformation. The other, designated gp120(R), is directed to "continuous" epitopes whose antigenicity is not affected by the reducing agent, dithiothreitol (DTT). Figure 1 shows the detection of gp120(NR) antibodies in 19 of the 19 HIV-seropositive samples tested. Among the same 19 HIV seropositive samples, only seven had appreciable level of gp120(R) antibodies. This observation suggests that gp120(NR) antibodies are directed to antigenic epitopes that are more immunogenic than those recognized by gp120(R) antibodies, and that only some HIV-seropositive individuals have detectable gp120(R) antibodies.

gp120(R) Antibody and Clinical Manifestation

The observation that not all HIV-seropositive individuals have gp120(R) antibody raised the question of whether this type of gp120 antibody is correlated with clinical manifestations of HIV infection. To address this question, we tested 137 coded serum samples from HIV seropositive individuals who, according to the Walter Reed Staging Classification System (5), were at various clinical stages of HIV infection. As shown in Figure 2, the prevalence rate of gp120(R) antibody decreases as clinical conditions progress from asymptomic states to full-blown AIDS. This cross-sectional study suggests that the lack of gp120(R) antibody is associated with poor clinical outcome ($\text{chi-square}_{\text{MH-ext}} = 17.36$; $p=0.00003$).

Antigenic Epitopes Recognized by gp120(R) Antibody

The carboxyl portion of gp120 appears to contain antigenic epitopes that are recognized by gp120(R) antibody. This is supported by the observation that a gp120 fragment cleaved by the V8 protease is reactive only with those sera which have gp120(R) antibody reactivity (data not shown). This V8 protease generated peptide is mapped to the carboxyl portion of gp120 by its reactivity with three site-specific sera. Another line of evidence is that almost all sera with gp120(R) antibody reactivity recognize a gp120 recombinant peptide, designated gp120(343c), which covers the carboxyl one-third of the gp120 sequence.

Disease Progression and gp120(343c) Antibody

A retrospective follow-up study was conducted to determine if gp120(343c) antibody level is associated with disease progression. Our study population was selected from the Multi-Center AIDS Cohort Study (MACS) (6). The MACS group enrolled 4955 homosexual and bisexual males from four US cities between April 1984 and March 1985 who were at least 17 years old and did not have prior diagnosis of AIDS. By December 1, 1986 there were 88 individuals from this cohort who progressed to non-Kaposis AIDS. For each of the 88 AIDS cases, 5 center-matched controls, who were seropositive at enrollment and did not have AIDS by December 1, 1986, were randomly selected. All together, 383 controls were included in our study (Figure 3). In one center, there were not enough HIV seropositive controls for every AIDS case included in our study. This accounts for most of the missing subjects in the control group. Among these 383 controls, 292 had a T4 count greater than or equal to 400 at the time of enrollment and continued to have a T4 count greater than or equal to 400 at the end of the follow-up.

We tested our hypothesis that a lower level of gp120(343c) antibody was more likely to be found in the seropositive individual who progressed to AIDS than those

who did not have a sharp drop in their T4 counts and remained free of AIDS. Sera taken during the first visit for all 380 study subjects were assayed for their antibody reactivity to gp120(343c) peptide by immunoblot. Antibody binding reactivities to gp120(343c) peptide was scored by densitometry and ranked on a linear scale. As shown in Table 1, there is a statistically significant difference in the gp120(343c) antibody level between these two groups. In contrast with the antibody reacting to gp120(343c), comparable level of gp120(NR) antibody was found between these two groups (data not shown, Wilcoxon rank sum test, $p=0.9448$).

Conclusion

Our cross-sectional study reveals that HIV seropositive individuals who were at later stages of infection were less likely to have gp120(R) antibody. Our retrospective study shows that HIV seropositive individuals who subsequently developed AIDS had a lower level of gp120(343c) antibody than those who remained free of AIDS. Taken together, these results suggest that some naturally produced gp120 antibodies may influence the clinical outcome of HIV infection. At present, we cannot provide a definitive answer to the question of whether the lack of gp120(R) antibody or gp120(343c) antibody is the cause or a marker of disease progression. A more definitive answer will only come from prospective studies.

It should also be pointed out that both disease progressors and non-progressors in our retrospective study had comparable levels of gp120(NR) antibody. This observation suggests that this class of gp120-binding antibodies may not have significant role in preventing disease progression. In light of the observation that antigenic domains recogized by gp120(NR) antibodies are immunogenically more dominant than those recognized by gp120(R), the possibility should be considered that a gp120 molecule in its most native conformation may not be the best choice for eliciting strong antibody response to gp120(R) or gp120(343c) epitopes in the high risk population.

Section 2. Human Immunodeficiency Virus Type 1 has Additional Coding Sequence in the Central Region of the Genome

Human immunodeficiency virus type 1 (HIV-1), formerly called HTLV-III/LAV, or ARV, is the causative agent of acquired immune deficiency syndrome (AIDS) and related disorders (7-10). HIV-1 is an exogenous non-oncogenic retrovirus with a complex genomic organization. In addition to the three genes (gag, pol and env) commonly found in replication-competent animal retroviruses, five additional genes have been identified. Three of them, 3'orf, sor, and R,were initially predicted from DNA sequence analysis (11-14) and have been shown to encode products which induce antibody responses during natural infection (15-18). The 3'orf gene encoded p27 has characteristics of a GTP binding protein and was reported be a negative regulator of virus replication (19-21). The sor gene encodes a 23-kDa protein and HIV-1 sor mutants were known to have impaired infectivity (22,23). The presence of an R gene was suggested by the observation that sera from HIV-1 infected individuals contained antibody reactivity to a recombinant R protein (18). The natural product and the function of R remain unknown. The tat and art/trs genes were identified from functional studies (24-27). They are composed of three exons; their second coding exons overlap the env gene but use different reading frames. Both tat and art/trs genes are requisite for viral replication (26-29). The tat product is a 14-kDa protein which regulates expression of viral genes at the transcriptional and/or post-transcriptional level (30-35). The art/trs gene encodes a product of about 19-kDa which is required for the expression of virus structrual proteins (26,27,36).

Despite the unusually large number of genes involved in the life cycle of HIV-1, there are many other open reading frames in the genome which may be considered candidate regions to encode for additional virus proteins. For instance, a small open reading frame (orf) flanked by the tat and art/trs first coding exons and the env

gene is conserved among several HIV-1 isolates (37). This orf, termed U in one previous report, has the potential to encode a protein of about eighty amino acids (38). In this study we present evidence to show that orfU is another coding sequence of HIV-1, and its product induces an antibody response during natural infection.

Figure 4 shows the HIV-1 DNA sequence in the orfU which was cloned into the vector pXVR, and the steps involved in the construction of plasmid pUSS19. The reading frame in the junction region between preceeding v-ras^H and the HIV-1 DNA insert was further verified by DNA sequencing (Fig. 4). The recombinant protein expressed by pUSS19 is expected to contain the first 111 amino acids of v-ras^H, the last fourty-nine amino acids of orfU, and one extra glycine residue at the junction between the preceeding v-ras^H and the HIV-1 insert DNA.

Upon induction by IPTG, a recombinant protein of about 20-kDa was detected in the lysate of E. coli carrying pUSS19 (Fig. 5). Because of its relative insolubility, partial purification of the protein, designated recombinant U peptide in this report, can be readily achieved (Fig. 5). The recombinant U peptide was specifically recognized by a monoclonal antibody to v-ras^H p21 and was not detected in lysate of uninduced cells by Western blotting analysis (data not shown). The expression level was markedly diminished when the recombinant U peptide was expressed without fusion.

The question of whether the HIV-1 DNA insert in pUSS19 contains a coding sequence of HIV-1 was addressed by screening HIV-1 antibody positive sera for antibody reactivities to the recombinant U peptide. Representative Western blotting profiles are shown in Figure 5. Among the 136 HIV-1 antibody positive sera tested, 43 had detectable antibody to the recombinant U peptide (lanes 7-9 in Fig. 5B, C). No reactivity to the recombinant U peptide was detected with sera from 20 HIV-1 antibody negative blood donors (lanes 1-3 in Fig. 5B, C) nor with 27 HIV-2 antibody positive sera. Reactivity to the recombinant U peptide was unlikely to be directed to

the v-ras^H portion of the fusion peptide since no reactivity with v-ras^H p21 expressed by plasmid pXVR was detected in 20 randomly selected sera that tested positive for antibodies to the recombinant U peptide. These results indicate that at least some part of the HIV-1 sequence in the pUSS19 is expressed during natural infection.

A cross-sectional study was conducted to determine if antibody to the recombinant U peptide is more likely to be detected at some particular clinical stages of HIV-1 infection. For this analysis, sera from 136 HIV-1 positive individuals whose clinical status had been previously staged using the Walter Reed (WR) clinical staging classification system were used (43). As shown in Fig. 3, the distribution of antibody to recombinant U peptide appears to be bi-modal with one peak associated with early stages (WR2 and WR3) of infection and the other associated with the late stage (WR6) of infection. The elevation of antibody prevalence in stage 6 is unique when compared with the prevalence of antibodies to other viral antigens, such as gag gene encoded p24, sor gene encoded p23, and 3'orf encoded p27, all standardized with the same panel of sera (Fig. 6).

Our data provide evidence for the presence of another coding sequence located in the central region of the HIV-1 genome. The exact initiation codon of this new HIV-1 gene remains to be determined. One possibility is that the first methionine codon found after a known splice acceptor site [nucleotide number 5557 according to Muesing et al. (14)] of HIV-1 is the initiator. This putative initiator codon is conserved in the orf U in several HIV-1 isolates (37). We have detected an HIV-1 specific protein of about 16-kDa in Hut78 cells transfected with an HIV-1 infectious clone, HX10, which has an initiation codon ATG in the orf U (47), using a goat serum raised against the recombinant U peptide (unpublished observation). The molecular weight of this protein is larger than the size predicted from the sequence of the orf U. This raises the possibility that either a more complex splicing pattern is involved

in the expression of orf U or the native U protein undergoes extensive post-translational modification. However, if the latter explanation is correct, it suggests that the product of this gene is dispensable for viral replication, because an infectous clone HXB2 (47) lacks the putative initiation codon of orf U.

In the natural course of infection, HIV-1 is more readily isolated from patients with AIDS (WR6) (R. Redfield, unpublished observation). Such patients showed a higher prevalence of antibody to the recombinant U peptide. In agreement with the elevated rate of recombinant U peptide antibody in patients at the late stage of HIV-1 infection, one seropositive patient that was sequentially analyzed developed recombinant U peptide antibody 12-18 months prior to the development of AIDS. This antibody reactivity persisted throughout the follow-up (Fig. 7). These observations raise the possibility that antibody to the recombinant U peptide is developed during and/or after some active rounds of viral replication.

The lack of antibody reactivity to the recombinant U peptide in 27 HIV-2 positive sera tested is consistent with the observation that the HIV-1 coding sequence identified in this study has no counterpart in HIV-2 (48). This feature should allow the use of antibody reactivity to the U peptide to help confirm infections by HIV-1 related viruses among West Africans whose sera have antibody reactivity to both HIV-1 and HIV-2 (49).

Conclusion

Eight coding regions designated gag, pol, env, sor, R, tat, art/trs, and 3'orf, have been identified in the genome of the human immunodeficiency virus type 1 (HIV-1). Several other open reading frames have the potential to encode additional viral proteins. In this study, we show that HIV-1 has another coding sequence whose product is expressed during natural infection. Unlike antibody to other HIV-1 proteins, the prevalence of antibody to the product encoded by this new region is elevated in patients with AIDS. Because no analogous coding region has been identified in HIV-2, the antibody to the product of this coding region may serve as a marker to distinguish infection with HIV-1 from infection with HIV-2.

Procedures and reagents used in this study are described as follows:

Plasmids

All DNA manipulation was according to the standard techniques (39). Plasmid pRTUA (Fig. 4) was constructed by cloning a 2190 base-pair Kpn I-Kpn I fragment from the HTLV-III clone BH10 into pUC18 (12). A 194 base-pair Ssp I - Sma I fragment from pRTUA was ligated with Nco I linkers d(CCCATGGG) (New England Biolabs), and then cloned into the Nco I site of a vector pXVR (40). After transfecting E. coli X-90 [ara D(lac-pro) nalA argE(Am) thi Rif^r $F'(lac^+$-pro^+ $lacI^{q1})$] by the calcium chloride method, clones with insert DNA in the right orientation were selected by restriction mapping (41). One of these clones, pUSS19, was used in this study. For DNA sequencing, a Hind III- Kpn I fragment of pUSS19 which contains the upstream junction region between v-ras^H and the HIV-1 DNA insert was cloned into phage M13 and sequenced by the chain termination method (42).

Sera

Serum specimens used in this study included 137 sera from HIV-1 infected individuals whose clinical status had been previously established by the Walter Reed (WR) clinical staging system (43). Antibody reactivity to the recombinant peptide expressed by pUSS19 was determined in 136 sera because one sample did not have sufficient quantity. Twenty-seven HIV-2 positive sera from West Africans, and 20 HIV-1 negative sera from healthy blood donors were also included in this study. A monoclonal antibody to v-ras^H p21 was a gift of Dr. B.T. Pan of Harvard Medical School. Some sequential serum samples from HIV-1 seropositive people were kindly provided by the NIAID funded Multi-Center AIDS Cohort Study (44).

Antigen preparation and immunodetection

An overnight culture of E. coli X-90 carrying plasmid pUSS19 was innoculated into the Luria-Bertani medium supplemented with 50 mg/ml of ampicillin at a 1:20 dilution. Induction with 5mM isopropyl-b-D-thiogalactopyranoside (IPTG) was

started when OD550 reached 0.25. After growing the cells at 37°C with vigorous aeration for 3 hrs, cells were pelleted (4200rpm, 20min. at 4°C, Beckman Accuspin FR) and washed once with buffer containing 10mM Tris-Cl, pH7.5, and 50mM NaCl. After washing, the cell pellet was resuspended in buffer containing 0.1M DTT, 0.08M Tris-Cl, pH6.8, 2% $NaDodSO_4$, 10% glycerol, and 0.001% bromophenol blue (whole cell lysate) and separated on 13% $NaDodSO_4$ polyacrylamide gel electrophoresis (PAGE). $NaDodSO_4$ PAGE was done according to the Laemmli method (45). The procedure for partial purification of the protein expressed by pUSS19 was modified from that of Pallas et al. (41). Pelleted cells were resuspended in 20 times the cell volume of bacterial lysis buffer (25mM Tris-Cl, pH8.0, containing 1mM phenylmethylsulfonyl fluoride) and sonicated for six 45 seconds on ice. The sonicated suspension was centrifuged for 5 min at 13,000g, at 4°C. The pellet was washed sequentially with bacterial lysis buffer, bacterial lysis buffer containing 1% nonidet P-40 and 2mM-b-mercaptoethanol, and bacterial lysis buffer containing 2M NaCl and 2mM-b-mercaptoethanol. After each wash, the pellet suspensions were sonicated for 1 minute and centrifuged at 13000g, at 4°C for 5 min. The pellet was then resuspended in 25mM Tris-Cl (pH 8.0) containing 3M urea and incubated on ice for 1 hour. After sonication for 1 minute, the insoluble fraction (partially purified recombinant peptide) was separated on $NaDodSO_4$ PAGE. After electrophoresis, proteins were transferred to the nitrocellulose membrane (Schleicher and Schuell) by passive transfer. The amount of recombinant U peptide on each strip is about 0.25 mg. The procedures for Western blotting analysis was described previously (46).

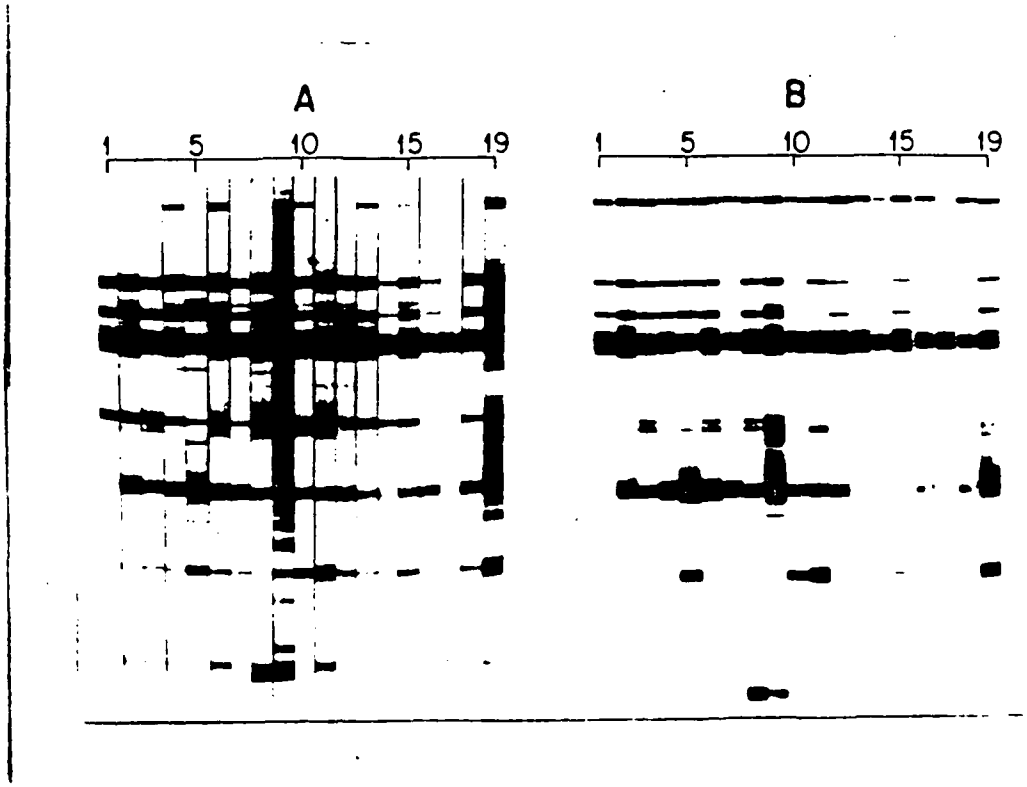
HIV-1 antigens used for Western blotting analysis in this study were prepared from culture supernatant of HTLV-III_B infected Molt-3 cell line (8,16). The presence of antibody against HIV-1 viral antigens was also determined by radioimmuno-precipitation and $NaDodSO_4$ PAGE as described previously (16). For this assay the cell lysates prepared from HIV-1 infected Molt-3 cells labeled with L-[^{35}S]-cysteine (>22.2TBq/mmol) or L-[^{35}S]-methionine (>29.6Tbq/mmol) (New England Nuclear) were used as antigens.

Table 1: Relationship Between Antibody to Recombinant Peptide gp120(343c) and Disease Progression

	gp120 (343c) Antibody Level						
Group	1	2	3	4	5	6	Total
Progressor	24	27	17	13	6	1	88
Non-progressor	41	65	63	59	54	10	292
Total	65	92	80	72	60	11	380

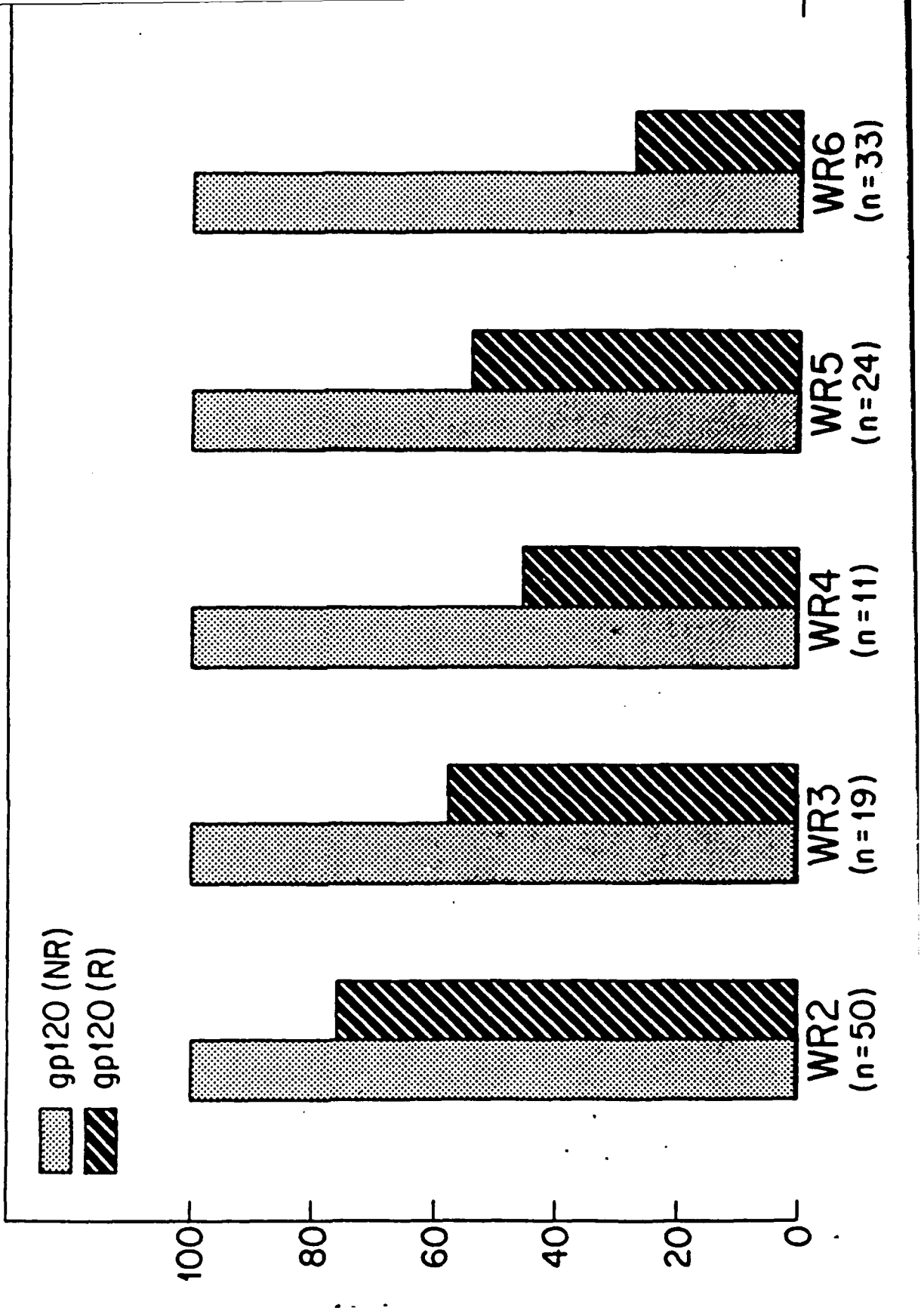
$P = 0.4 \times 10^{-4}$ (Wilcoxon Rank Sum Test)

Figure 1



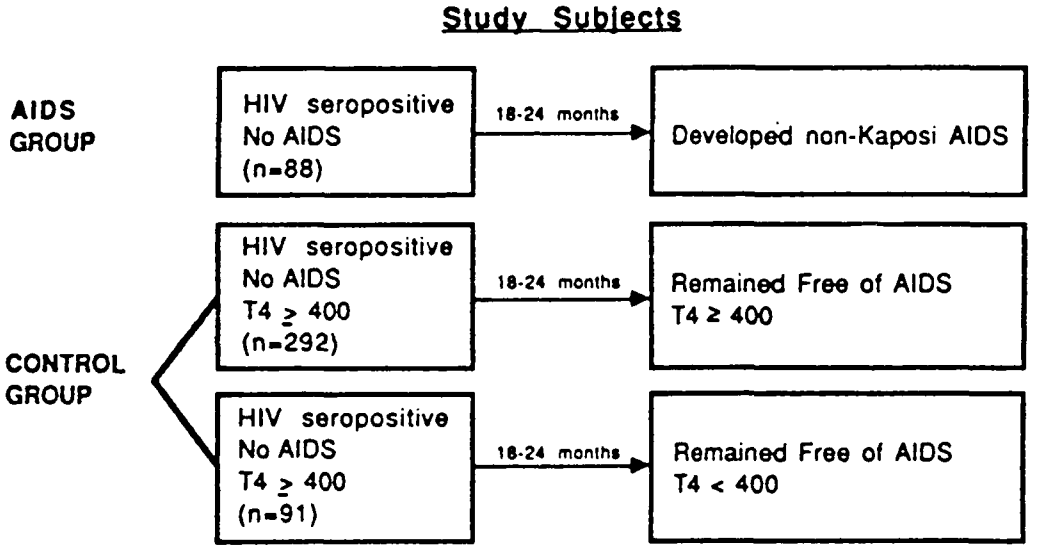
Detection of gp120(R) and gp120(NR) antibodies in sera of HIV-infection individuals. Nineteen serum samples known to have gp120 antibody reactivity by radioimmunoprecipitation assay were analyzed by immunoblotting for gp120(R) and gp120(NR) antibodies. Cell-free virions from HTLV-III$_B$ infected Molt/III cells were lysed with a buffer containing 0.15 M NaCl, 0.05 M Tris-HCl pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. For detection of gp120(R) antibody (panel A) equal volume of buffer containing 4% SDS, 0.16 M Tris-HCl pH 6.8, 20% Glycerol, 0.4% bromophenol blue, and 0.2 M Cleland's reagent were added to viral lysate before it was boiled at 100^o C for three minutes. For detection of gp120(NR) antibody (panel B), Cleland's reagent was not added to the buffer. Sera were tested at 1:200 dilution.

Figure 2



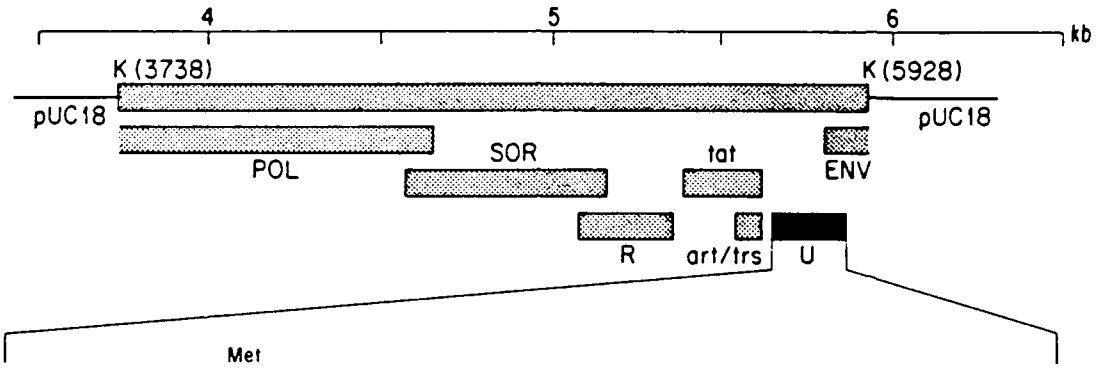
Prevalence of gp120(R) and gp120(NR) antibody among 137 HIV-seropositive patients whose clinical conditions have been classified according to the Walter Reed Staging Classification System. Walter Reed Stage 2 is abbreviated as WR2.

Figure 3



Retrospective follow-up study of 417 HIV-seropositive homosexual males enrolled in the Multi-Center AIDS Cohort Study.

Figure 4A



Met

GTA CAT GTA ATG CAA CCT ATA CAA ATA GCA ATA GTA GCA TTA GTA

GTA GCA ATA ATA ATA GCA ATA GTT GTG TGG TCC ATA GTA ATC ATA

SspI (5736)

GAA TAT AGG AAA ATA TTA AGA CAA AGA AAA ATA GAC AGG TTA ATT

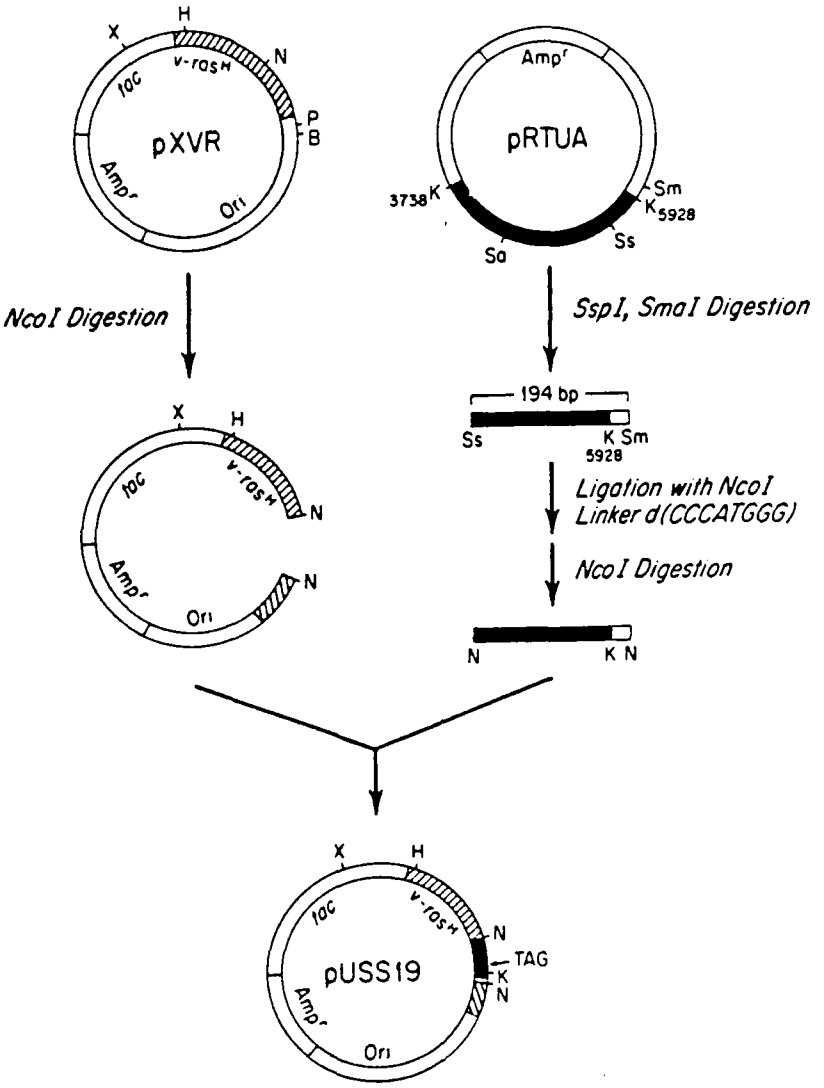
GAT AGA CTA ATA GAA AGA GCA GAA GAC AGT GGC AAT GAG AGT GAA

GGA GAA ATA TCA GCA CTT GTG GAG ATG GGG GTG GAG ATG GGG CAC

CAT GCT CCT TGG GAT GTT GAT GAT CTG TAG

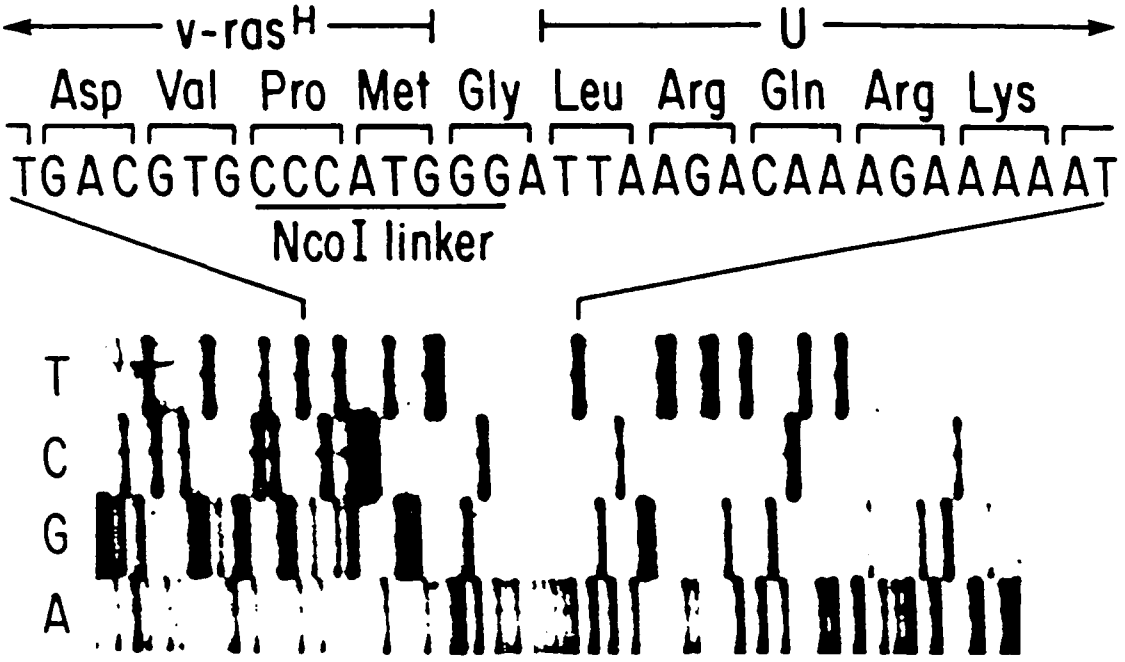
The location of the orfU and its relationship to the other genes of HIV-1 in plasmid pRTUA. Plasmid pRTUA contains a 2190 base-pair Kpn I-Kpn I fragment from HTLV-III DNA clone BH10 (12). The orfU sequence of BH10 is shown. The nucleotide number is according to that of Ratner et al. (12).

Figure 4B



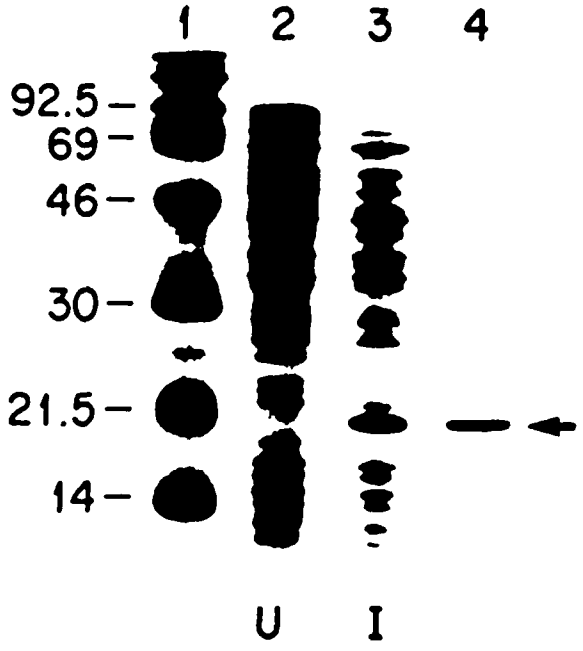
(B) Steps involved in the construction of pUSS19. A194 base-pair Ssp I - Sma I restriction fragment from plasmid pRTUA was ligated with Nco I linkers, and cloned into the Nco I site in pXVR (40). Amp^r, ampicillin resistance gene; tac, tac promoter; Ori, origin of replication; B, Bam HI; H, Hind III; K, Kpn I; N, Nco I; P, Pst I; Sa, Sal I; Sm, Sma I; Ss, Ssp I; X, Xba I; TAG, stop codon.

Figure 4C



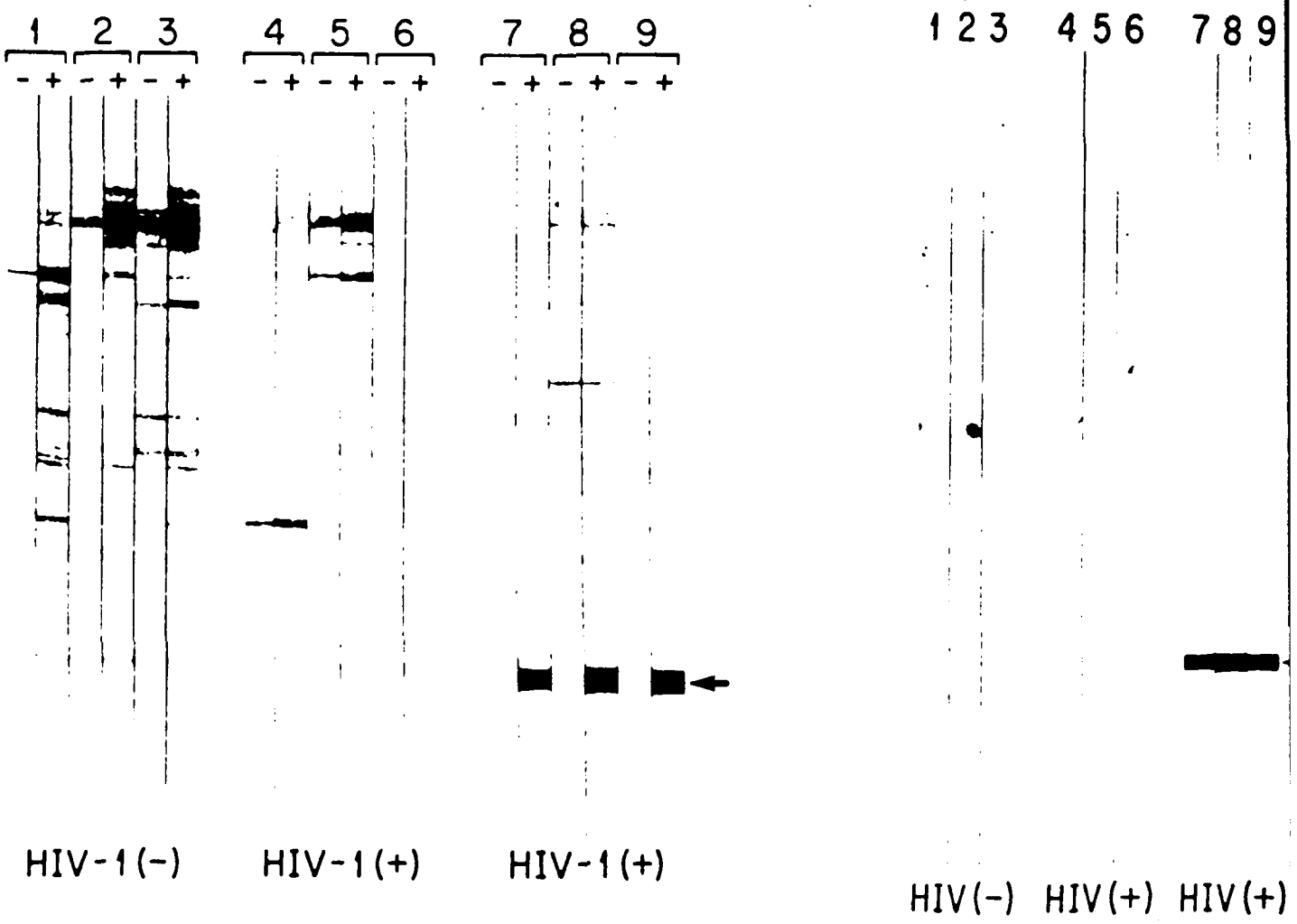
(C) The sequence of the junction between the preceeding v-ras^H and orfU was determined by the chain termination method (42). The deduced amino acid seqence is also shown. One extra glycine residue was introduced at the beginning of the orfU sequence in this construct.

Figure 5A



Expression of recombinant U peptide. NaDodSO4 PAGE analysis of whole cell lysates from IPTG induced X-90 culture (lane 3, indicated by I) and its uninduced control (lane 2, indicated by U). Lane 4 shows partially purified recombinant U peptide. Recombinant U peptide was indicated by the arrow. Molecular markers are shown in kDa (lane 1).

Figure 5B and 5C



(B) and (C) Antibody reactivity to the recombinant U peptide. Western blot analyses were carried out with whole cell lysate from uninduced controls and IPTG-induced cultures (B), and partially purified recombinant U peptide (C). Lanes 1, 2, and 3 are representative sera from seronegative blood donors. Lanes 4 to 9 are representative sera from HIV-1 seropositive donors. In (B), cell lysate from IPTG induced culture is indicated by +, and - indicates uninduced controls. In (C) all cell lysates were from induced cultures. The position of the recombinant peptide U was indicated by the arrow.

Figure 6

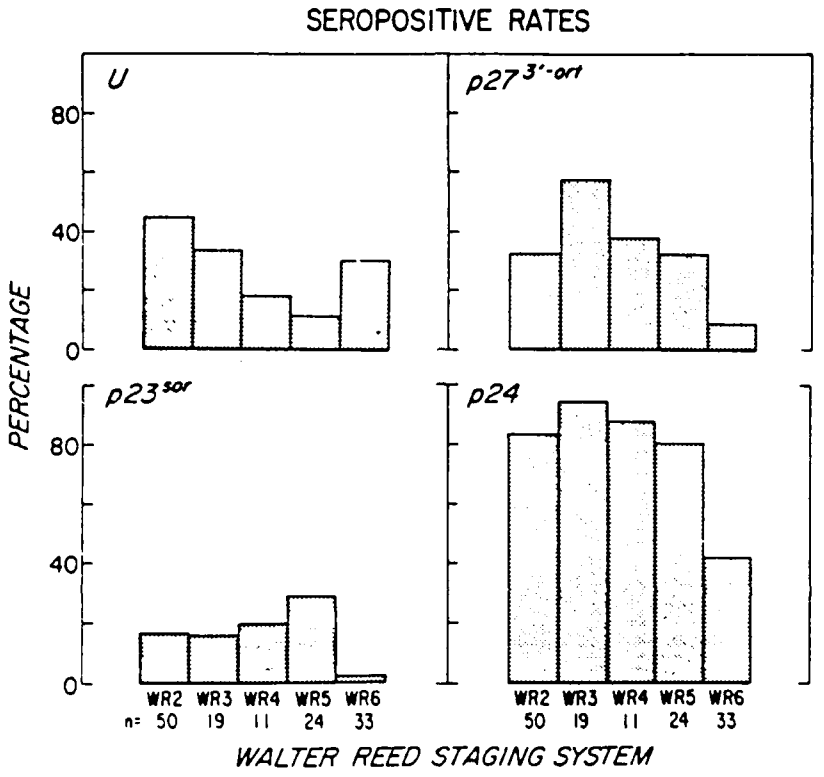


Figure 6. The prevalence of antibodies to recombinant U peptide, p27, p23 and p24 in different clinical stages of HIV-1 infection. The vertical axis shows the prevalence rates of antibodies to recombinant U peptide, p27, p23 and p24. The horizontal axis shows 5 Walter Reed clinical stages. Antibody to recombinant U peptide was analyzed by the Western blot technique and antibodies to other HIV-1 antigens were scored by radioimmunoprecipitation and NaDodSO4 PAGE assay. The number (n) of patients in each clinical stage is indicated below each column. One serum from a patient classified at Walter Reed stage 3 did not have sufficient quantity for assaying antibody reactivity to recombinant U peptide.

Figure 7

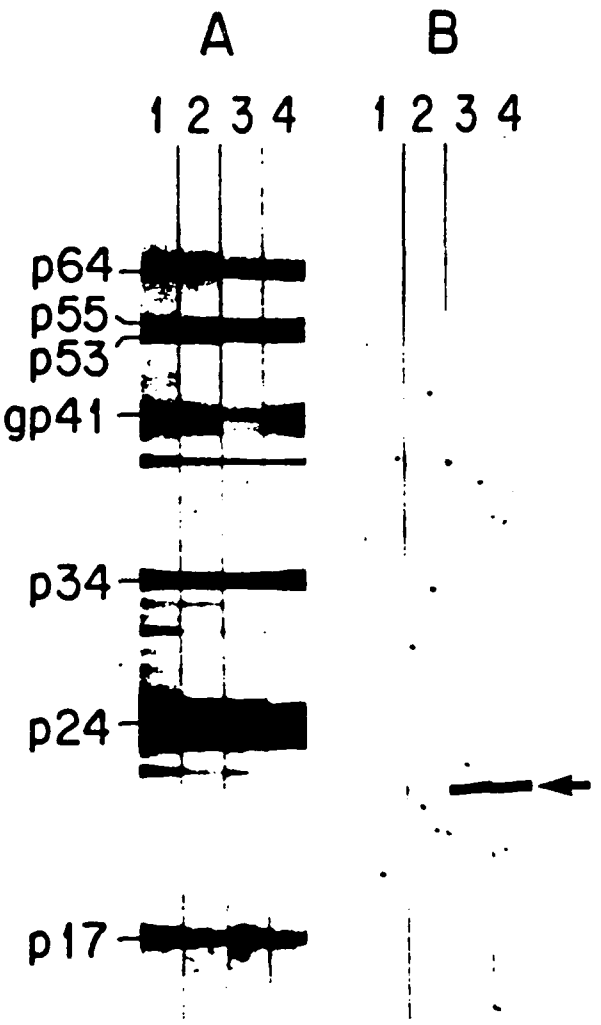


Figure 7. Antibody reactivity to the recombinant U peptide detected in sequential serum samples from an HIV-1 seropositive patient. The antigen used in panel A was HIV-1 viral lysate prepared from the supernatant of HTLV-IIIB infected Molt-3 cell line. The antigen used in panel B was partially purified recombinant U peptide. Antibody reactivities of four sequential serum samples taken at 6 months intervals are shown in sequence from lane 1 to lane 4. The patient was diagnosed with AIDS at the visit when the serum specimen shown in lane 4 was collected.

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