

TSE. In either case, the addition of glucocorticoids and GR then results in decreased transcription, demonstrating interference with the activity of the CRE and CRE-binding protein. The presence of GR binding sites that overlap both CREs supports a model in which negative control is exerted through direct GR binding to DNA sequences that overlap the CREs, with consequent blockage of CRE activity. This mechanism of interference between transcription factors may apply not only to negative regulation by steroid receptors but also to negative regulation in other systems.

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Abnormalities in Structure and Expression of the Human Retinoblastoma Gene in SCLC

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Small cell lung cancer (SCLC) has been associated with loss of heterozygosity at several distinct genetic loci including chromosomes 3p, 13q, and 17p. To determine whether the retinoblastoma gene (*Rb*) localized at 13q14, might be the target of recessive mutations in lung cancer, eight primary SCLC tumors and 50 cell lines representing all major histologic types of lung cancer were examined with the *Rb* complementary DNA probe. Structural abnormalities within the *Rb* gene were observed in 1/8 (13%) primary SCLC tumors, 4/22 (18%) SCLC lines, and 1/4 (25%) pulmonary carcinoid lines (comparable to the 20 to 40% observed in retinoblastoma), but were not detected in other major types of lung cancer. *Rb* messenger RNA expression was absent in 60% of the SCLC lines and 75% of pulmonary carcinoid lines, including all samples with DNA abnormalities. In contrast, *Rb* transcripts were found in 90% of non-SCLC lung cancer lines and in normal human lung. The finding of abnormalities of the *Rb* gene in SCLC and pulmonary carcinoids (both neuroendocrine tumors) suggests that this gene may be involved in the pathogenesis of a common adult malignancy.

IN SEVERAL CHILDHOOD TUMORS, INCLUDING retinoblastoma and Wilm's tumor, there is growing evidence to indicate that the inactivation of both alleles of certain genes triggers tumorigenesis (1-3). The genomic locus determining susceptibility to retinoblastoma has been mapped to chromosome 13q14 (4), and several groups have obtained complementary DNA (cDNA) clones derived from this region that detect a DNA segment with properties of the putative retinoblastoma (*Rb*) gene (5-7). Evidence for this gene being the site of recessive mutations leading to tumor formation in retinoblastoma is based on the finding of structural changes within the gene, including internal homozygous deletions in a number of retinoblastomas, and the presence of altered or absent messenger RNA (mRNA) expression in the majority of both sporadic and familial forms of the tumor.

Specific chromosomal deletions have now been reported in various adult tumors (8), suggesting that "recessive oncogenes" may be important in the pathogenesis of these malignancies. In the case of small cell lung cancer (SCLC), a consensus deletion of DNA in the region 3p14-3p21 has been identified in virtually all cases (9-11). Several studies have also identified nonrandom changes involving other chromosomes, including chromosome 13. For example, one

cytogenetic study of SCLC reported that chromosome 13 was the most frequently underrepresented chromosome, with 17 out of 21 SCLC lines having absent or hypodiploid numbers of chromosome 13 (12). In two recent studies, polymorphic probes from the long arm of chromosome 13 that spanned the region 13q12-13q33 demonstrated a reduction to homozygosity at one or more informative loci in primary tumor tissue from 18 of 23 patients with SCLC (11, 13). SCLC is an aggressive adult tumor which phenotypically resembles retinoblastoma in that both display properties of neural or neuroendocrine differentiation (14, 15) and both can have deregulated *N-myc* expression (16, 17). These observations, coupled with the recent report of structural abnormalities of the *Rb* gene in ~20% of osteosarcomas and other mesenchymal tumors (18), prompted us to examine the

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DNA and RNA status of the *Rb* gene in primary SCLC tumor tissue and in lung cancer cell lines to investigate the possible role of the *Rb* locus in the pathogenesis of these tumors.

Using the p0.9R and p3.8R cDNA probes (5), which represent the 4.7-kb *Rb* transcript that spans more than 180 kb of genomic DNA (6, 18), we analyzed DNA from eight SCLC primary tumor samples. We also studied DNA and RNA from 50 lung cancer cell lines (19), including 26 SCLC lines, 20 non-SCLC lines (eight adenocarcinomas, five large cell carcinomas, four bronchioloalveolar carcinomas, two adenosquamous carcinomas, and one squamous carcinoma), and four pulmonary carcinoid tumors (Table 1).

Structural rearrangements of the *Rb* gene were detected in DNA from one of the SCLC primary tumors (Fig. 1B, lane 10). Tumor DNA from this specimen exhibited novel Hind III fragments at 24, 16, and 11 kb. The 16-kb band was amplified several-fold relative to the other bands. In addition, the normal 10-kb band was markedly reduced in intensity, while the 7.5-, 6.2-, 5.5-, 4.5-, and 2.1-kb bands appeared normal. As the hybridization signal of the 10-kb fragment was much less than 50% of the expected intensity, this fragment may have been homozygously deleted in the tumor, with the residual band seen on DNA blot analysis representing contaminating normal cells in the biopsy sample. Similar abnormalities were also noted when the DNA was digested with Sst I (20). To expand on these observations and to examine correlations between DNA and RNA abnormalities, we studied the DNA and RNA status of *Rb* in lung cancer cell lines (Table 1).

There were structural abnormalities within the *Rb* gene in four SCLC cell lines and in one carcinoid line (Fig. 1). These structural changes were of two types: (i) the homozygous loss of a normal Hind III fragment, associated with the appearance of one or two novel-sized bands, and (ii) reduced intensity of one or more normal bands by at least 50%, confirmed by densitometry, suggesting the presence of hemizygous deletions. SCLC line H889 displayed a complete loss of the 7.5-kb fragment with the appearance of a new band at 10.6 kb as identified with the p3.8R probe (Fig. 1B, lane 3), while H187 had a complete loss of the 14.0-kb Hind III fragment associated with the appearance of two new bands of 12.0 and 2.8 kb as determined with the p0.9R probe (Fig. 1A, lane 4). Additionally, in the carcinoid line H679, the p3.8R probe detected a doublet at 2.25 and 1.95 kb in place of the normal 2.1-kb band (Fig. 1B, lane 7). In all three cases, these changes were

present in DNA obtained from the cell lines at different time points up to 28–50 passages in culture, suggesting that they were stable abnormalities. To exclude the possibility that the different patterns seen in H187, H679, and H889 represented Hind III restriction fragment length polymorphisms (RFLP), we digested DNA from these cell lines with Eco RI and Sst I, and by comparing to normal thymus DNA we observed rearrangements and homozygous loss of bands in these digests as well (20). In addition, 18 samples of normal human DNA probed with both p0.9R and p3.8R demonstrated a uniform pattern of restriction fragments with no Hind III polymorphisms (5), and no polymorphism has been reported for these restriction fragments among over 80 retinoblastomas and other tumors now examined. The homozygous internal deletion involving the 7.5-kb Hind III fragment, seen in line H889, has been observed in a large number of retinoblastomas and has been implicated as the possible site of a "mutational hotspot" (7).

SCLC lines H345 and H378 (Fig. 1, lanes 1 and 5, respectively) contained all expected Hind III bands detected by p3.8R, but on visual inspection both lines appeared to contain several bands of decreased intensity. To evaluate the possibility of partial *Rb* deletions on one allele in H345 and H378, densitometric analysis was performed to

quantitate the DNA band intensities in both lines. Reproducible densitometric tracings were obtained and quantitated on autoradiograph films of DNA blots (hybridized either with p0.9R or p3.8R) from H345 and H378, 13 other lung cancer cell lines with normal-appearing DNA patterns, a normal B-lymphoblastoid line, and a normal human thymus specimen (fresh uncultured tissue). For DNA blots hybridized with p0.9R, all lung cancer lines, including H345 and H378, displayed densitometric tracings very similar to those for the normal human DNA samples. For blots hybridized with p3.8R, the tracings for H345 and H378 were markedly abnormal (Fig. 2), while the 13 other lung cancer lines showed tracings similar to those for normal human DNA. To quantitate these abnormalities, the areas under the peaks corresponding to each of the Hind III bands detected by p3.8R were calculated and normalized to the 10.0-kb peak, which served as an internal control. Each of the 13 lung cancer lines with normal densitometric tracings had quantitative estimates of band intensity ratios similar to those for the normal DNA samples, with minimal variability. In H345, however, there appeared to be a decrease in intensity by approximately 50% of the 6.2-kb band, suggesting that an internal hemizygous deletion of this genomic fragment may have occurred. In H378, the abnormalities were

Table 1. Summary of DNA and RNA status of the *Rb* gene in 50 lung cancer lines.

Cell line*	DNA changes	RNA expression†	Cell line (histology)	DNA changes	RNA expression
<i>Small cell lung cancer</i>			<i>Pulmonary carcinoids</i>		
H187	5' rearrangement	–	H679 (Atypical)	3' rearrangement	–
H345	3' rearrangement	–	H720 (Atypical)	None	–
H378	3' rearrangement	–	H835 (Atypical)	None	–
H889	3' rearrangement	–	H727 (Typical)	None	+
H82	None	–	<i>Non-small cell lung cancer</i>		
N417	None	–			
H510	None	–	H125 (Adenosquamous)	None	+
H524	ND	–	H226 (Squamous)	None	+
H526	None	–	H322 (Bronchioloalveolar)	None	+
H735	None	–	H358 (Bronchioloalveolar)	None	+
H748	ND	–	H460 (Large cell)	None	+
H774	None	–	H522 (Adenocarcinoma)	None	+
H1284	None	–	H661 (Large cell)	None	+
H1304	None	–	H810 (Large cell)	None	+
H1450	ND	–	H820 (Bronchioloalveolar)	None	+
H69	None	tr	H838 (Adenocarcinoma)	None	+
N592	None	tr	H1373 (Adenocarcinoma)	None	+
H711	ND	tr	H1385 (Large cell)	None	+
H847	None	tr	H1404 (Bronchioloalveolar)	None	+
H1622	None	tr	H1435 (Adenocarcinoma)	None	+
H209	None	+	H1437 (Adenocarcinoma)	None	+
H841	None	+	H23 (Adenocarcinoma)	None	tr
H1092	None	+	H1355 (Adenocarcinoma)	None	tr
H1105	None	+	H596 (Adenosquamous)	None	–
H1184	None	+	H1155 (Large cell)	None	–
H1436	None	+	H1445 (Adenocarcinoma)	None	ND

*Full designation of the cell lines includes the prefix "NCI-" †+, easily detectable transcript (comparable to normal lung expression); tr, trace; –, absent; ND, not determined.

more complex but included a decrease in intensity of about 50% for the 6.2-kb and 2.1-kb bands, consistent with a hemizygous deletion of the 3'-most end of the gene (see Fig. 1 for genomic structure). The homozygous and hemizygous DNA structural abnormalities described above are similar to those recently published for retinoblastoma and associated mesenchymal tumors, and the presence of such changes has been interpreted as evidence to implicate the *Rb* locus as the target of recessive mutations in these tumors (5-7).

Total and poly(A)-selected RNA were prepared from the lung cancer lines and RNA analysis was performed with the p3.8R probe (Table 1 and Fig. 3). Of 26 SCLC lines, 15 expressed no detectable *Rb* mRNA, five expressed trace levels, and six had easily detectable levels of the *Rb* transcript. In addition, three of four pulmonary carcinoids expressed no detectable *Rb* transcript. Pulmonary carcinoid tumors are similar to SCLC in that both contain neurosecretory granules and exhibit neuroendocrine differentiation. Thus, these two tumors are distinctive from other types of lung cancer and may represent variants of malignant transformation in the same neuroendocrine precursor cell (21). All three tumors lacking the transcript were characterized as "atypical" carcinoids, with clinical and histologic features intermediate between SCLC and classic carcinoid (14). In contrast to SCLC and carcinoid, 15 of 19 non-SCLC lines

expressed abundant steady-state levels of the 4.7-kb transcript, two expressed trace amounts, and two had undetectable levels of the *Rb* mRNA. These data demonstrated a correlation between the DNA and RNA status (Table 1) in that the four SCLC lines and one carcinoid line with gross structural changes within the *Rb* locus expressed no detectable *Rb* transcript, while the SCLC lines which did express abundant levels of mRNA, and all of the non-SCLC lines, had normal DNA patterns on DNA blots. Eight SCLC lines, however, expressed no detectable mRNA but had normal DNA patterns with the probes used in this study. As postulated for retinoblastoma, some or all of these cases may have contained small mutations in the *Rb* gene which accounted for the absent mRNA but which were undetectable by our DNA analysis.

Expression of *Rb* mRNA was detected in total RNA from normal adult human lung (Fig. 3A, lane 1) and poly(A)-selected RNA from bovine lung (20). Although the cell of origin of SCLC remains controversial, the finding of *Rb* gene expression in lung tissue, as well as in a few SCLC lines and in all other normal tissues examined thus far (6), suggests that *Rb* may normally be expressed in most cells, including the SCLC precursor cell. Therefore, the lack of expression in the SCLC and carcinoid lines supports the idea that the *Rb* gene has been inactivated in these lines.

In light of our finding of DNA and RNA

abnormalities in these cell lines, we analyzed the karyotypes of 11 of the SCLC lines for which adequate metaphase spreads could be obtained, looking for evidence of alterations specifically involving chromosome 13. All three of the SCLC lines with detectable rearrangements of the *Rb* gene that were examined cytogenetically contained abnormalities involving the region 13q14. H889 displayed a del(13)(q14>ter) and had no normal copy of chromosome 13. H187 contained a t(8;13)(q24;q14) and a t(9;13)(q13;q14), and H378 contained a del(13)(q14>ter) and a t(3;13)(p21;q14). In H510, a cell line with no *Rb* RNA expression and a normal DNA blot pattern,

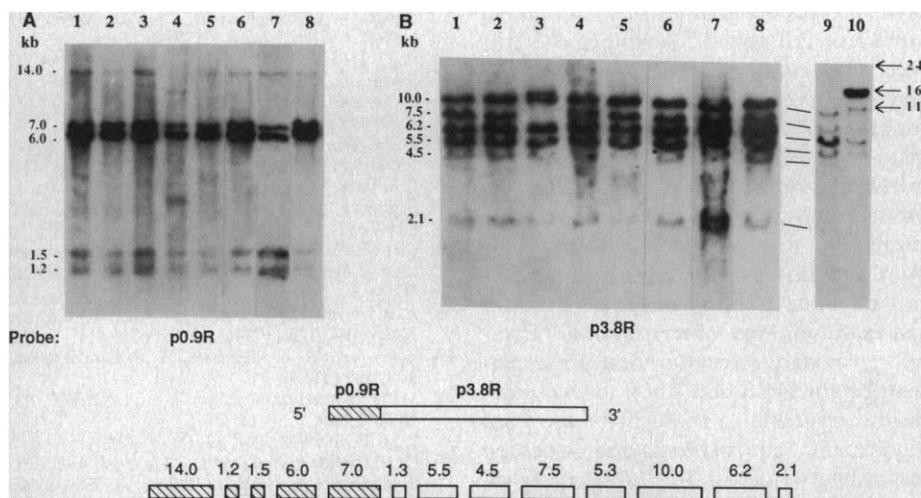


Fig. 1. Genomic restriction endonuclease analysis of the *Rb* in a representative panel of lung cancer lines. DNA (10 μ g) extracted from seven lung cancer cell lines (H345, H510, H889, H187, H378, H209, and H679; lanes 1 to 7, respectively), normal human thymus (lane 8), and two primary SCLC tumor specimens (lanes 9 to 10, panel B) were digested to completion with Hind III and transferred to nitrocellulose after 0.8% agarose gel electrophoresis. Filters were then hybridized with either the p0.9R (A) or the p3.8R (B) 32 P random-primed cDNA probes for 18 hours under standard hybridization conditions (26). For p0.9R, a 675-bp Eco RI-Hpa I fragment was used as a probe since this removed a GC-rich region that conferred a high background on hybridization. Filters were washed twice at room temperature in $2 \times$ SSC/0.1% SDS for 30 minutes and then twice in $0.1 \times$ SSC/0.1% SDS at 55°C for 1 hour. A diagram at the bottom of the figure depicts the previously described *Rb* cDNA clone and the Hind III genomic fragments (5, 6) detected by the p0.9R probe (hatched boxes) and the p3.8R probe (open boxes).

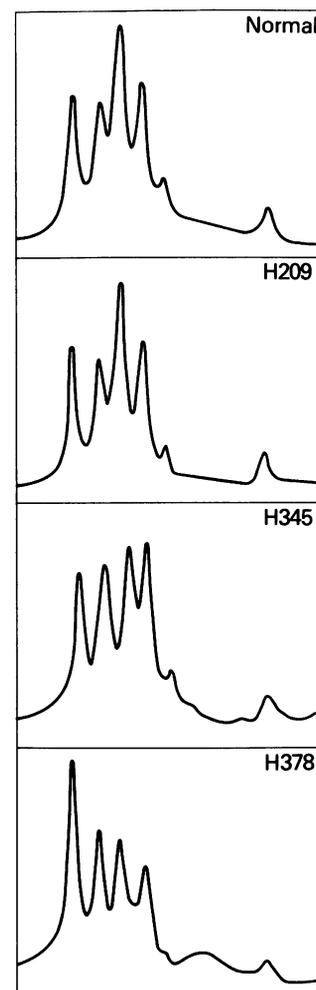


Fig. 2. Densitometric analysis. The top panel shows the densitometric tracing observed with normal human DNA (from fresh thymus tissue) digested to completion with Hind III and hybridized with the p3.8R probe. The five peaks from left to right represent, respectively, the Hind III fragments at 10, 7.5, 6.2, 5.5, 4.5, and 2.1 kb (see Fig. 1B). The tracings were obtained on a Hoefer scanning densitometer (model GS300). Thirteen tumor lines, including H209 shown above, demonstrated densitometric patterns similar to that obtained for normal human DNA. In contrast, cell lines H345 and H378, shown in the lower two panels, had anomalous patterns.

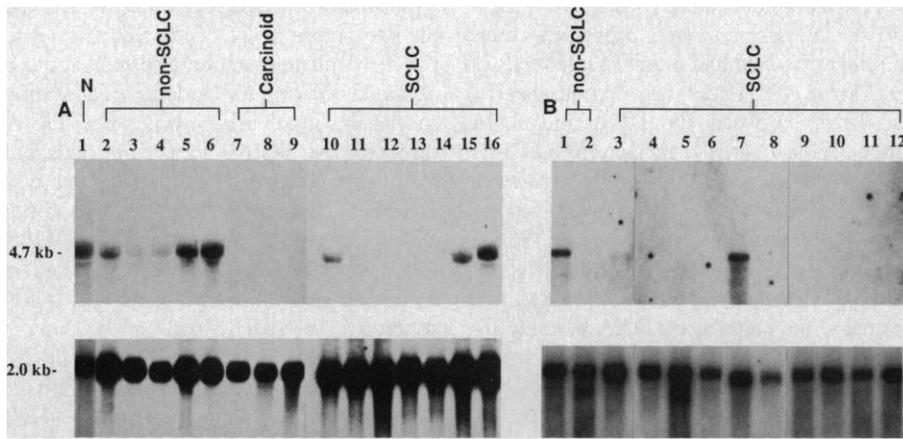


Fig. 3. *Rb* gene expression in a representative panel of lung cancer lines. (A) Total RNA (15 µg) was extracted (27) from normal lung tissue obtained from a surgical specimen (lane 1), five non-SCLC lines (H820, H1355, H1373, H1404, and H1437; lanes 2 to 6), three carcinoid lines (H720, H835, and H679; lanes 7 to 9), and seven SCLC lines (H1092, H1622, H748, H1304, H774, H1436, and H1105; lanes 10 to 16). (B) Two micrograms of poly(A)-selected RNA (28) was extracted from two additional non-SCLC lines (H125 and H23; lanes 1 and 2) and ten additional SCLC lines (N592, H345, H378, H510, H209, H187, H526, H524, H82, and N417; lanes 3 to 12). The above RNA samples were transferred to nitrocellulose after formaldehyde-denaturing gel electrophoresis (29) and filters were hybridized sequentially with the ³²P random-primed p3.8R probe which detects the 4.7-kb *Rb* transcript (5–7) or with a β-actin cDNA probe which detects a 2-kb transcript (30). Autoradiographs were developed on XAR-5 film after 5 days at –70°C for p3.8R and after overnight exposure for β-actin. N, normal lung.

a portion of chromosome 13 was attached at band q14 to an unidentified chromosomal fragment, and there was no normal copy of 13. Two other cell lines with trace or absent *Rb* expression (H69 and H526) had lost one copy of chromosome 13 on the basis of expected modal number. In two SCLC lines with apparently intact structure and expression of *Rb* (H209 and H841) and in three lines with absent or trace expression (H82, N417, and H847) no abnormalities of number or structure of chromosome 13 were detected.

The presence of deletions within the *Rb* locus and lack of expression of the gene in SCLC is analogous to observations recently made in retinoblastoma and associated mesenchymal tumors where the gene is believed to contribute to tumorigenesis by a recessive “two-hit” mechanism (1). However, in contrast to retinoblastoma, which occurs in a familial form in 40% of cases (22), SCLC is not known to have a clear hereditary pattern. Although it is possible that an inherited abnormality of the *Rb* gene might predispose to SCLC (23), there is no established clinical association between retinoblastoma and SCLC, either in the same patient or within families. This would suggest that mutations at both alleles of the *Rb* gene in SCLC occur as somatic events. SCLC is closely associated with heavy cigarette smoking (24), and carcinogen exposure from tobacco may generate an increased mutation rate in the bronchial epithelium. Extremely large genes, such as *Rb*, may be especially susceptible to mutation and, thus, may com-

monly become inactivated in the bronchial epithelium of smokers.

From this study, it appears that abnormalities in structure and RNA expression of the *Rb* gene occur commonly in SCLC and pulmonary carcinoid but infrequently in other types of lung cancer. Further, the presence of DNA abnormalities of *Rb* in primary SCLC tumor tissue argues that these changes occur in vivo and are not an artifact of cell culture. Among the SCLC specimens examined, one primary tumor and 18% of cell lines demonstrated evidence for gross structural abnormalities of the *Rb* gene, and 60% expressed no detectable mRNA. Another 20% of SCLC lines expressed only trace amounts of the mRNA, suggesting that alterations in *Rb* gene expression may have occurred in these lines as well. Similar abnormalities of structure and expression were observed in our “atypical” pulmonary carcinoid lines. These percentages for DNA and RNA abnormalities were comparable to those that have been reported for retinoblastoma and associated mesenchymal tumors (5–7, 18). In comparison, no alterations of the *Rb* gene were detected in any of our non-SCLC lines, and 90% of these lines expressed *Rb* mRNA. Recent reports of chromosomal deletions at 3p in many non-SCLC tumors, as well as in SCLC (13, 25), suggest that the chromosome 3 deletion may be a common pathogenetic step to most or all major types of lung cancer. In contrast, a loss of heterozygosity of chromosome 13q markers appeared to occur mostly in SCLC when primary tumor

tissue was examined (11, 13), and our data support this observation in that abnormalities of the *Rb* gene were more specific to SCLC and “atypical” pulmonary carcinoid. The finding of abnormalities of the *Rb* gene in a large percentage of both of these types of pulmonary neuroendocrine tumors suggests that inactivation of this gene may be an important event in the pathogenesis of lung tumors of neuroendocrine differentiation. These findings indicate that the *Rb* gene may be involved in the pathogenesis of a common non-ocular adult malignancy, suggesting that it may play an important physiological role and may contribute to malignant transformation in various tissues besides the retina. Ultimately, functional studies will be required to prove a definitive role for this gene in the genesis of human tumors.

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Type-Restricted Neutralization of Molecular Clones of Human Immunodeficiency Virus

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In a study of the immunologic significance of the genetic diversity present within single isolates of human immunodeficiency virus type 1 (HIV-1), the neutralization of viruses derived from molecular clones of the HIV-1 strain HTLV-III_B by an extensive panel of sera was compared. Sera from HIV-1-infected patients and from goats immunized with polyacrylamide gel-purified HIV-1 envelope glycoprotein (gp120), native gp120, or gp120-derived recombinant peptides, showed marked heterogeneity in neutralizing activity against these closely related viruses. The change of a single amino acid residue in gp120 may account for such "clonal restriction" of neutralizing activity.

ONE STRATEGY FOR IMMUNOPROPHYLAXIS against acquired immunodeficiency syndrome is the administration of an immunogen intended to elicit protective virus neutralizing antibodies. Neutralizing activity (NA) against HIV-1 has been documented by many investigators using a variety of techniques (1-5). Domains within the HIV-1 envelope protein (gp120) (6-8) and the transmembrane glycoprotein (gp41) (9) have been shown to elicit or absorb NA. Sera from infected patients generally neutralize a broad range of isolates. In contrast, antisera produced in experimental animals by immunization with gp120 or recombinant or synthetic peptides display restricted capacity to neutralize isolates other than that from which the immunogen was derived (10-12). Recent observations indicate that individual isolates of HIV-1 are composed of populations of genetically and biologically distinct variants (13). To determine the implications of such heterogeneity for vaccine development and evaluation, we compared the susceptibility of three viruses molecularly cloned from HTLV-III_B (HIV-1/NIH/USA/1983/HTLV-III_B) to the neu-

tralizing effects of a number of antisera.

The molecularly cloned viruses designated HX10, HXB2, and HXB3, were obtained from a single cell line that was infected with HIV-1 (14, 15). They differ by less than 1% in nucleotide sequence, at 19 sites (24 amino acids) in the envelope region. Clones HXB2 and HXB3 were constructed by inserting fragments containing full-length proviral sequences into the expression vector pSP62, transfecting DH-1 bacteria, and recovering virus by protoplast fusion (16-18). HX10 was derived from the λBH10 phage proviral molecular clone (14), which lacks a 190-bp segment in the 5'-LTR, by inserting the 8.1-kb Cla I-Xho I fragment of λBH10

(which contains sequences coding for residue 14 of gag through residue 443 of the nef region) into pHXB2gpt2, and virus was recovered by protoplast fusion (17). The cloned viruses and the parent isolate replicate well in neoplastic T cell lines and possess similar infectivity and cytopathogenicity for CD4⁺ cells (17).

We initially examined the NA of a panel of 38 human sera from 25 seropositive patients against HTLV-III_B, HX10, HXB2, and HXB3. The NA of some sera against the different viruses differed (Table 1). Some neutralized one or two clones in high ($\geq 1:256$) titer although they showed low ($< 1:256$) NA against other clones (see Tables 1, HS-01 and HXB3, HS-12 and HX10, and HS-23 and HXB2); others neutralized all viruses to some degree (HS-06) or lacked appreciable NA against any virus

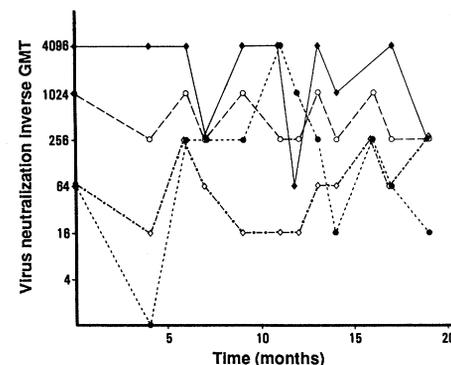


Fig. 1. Variation in neutralization of HTLV-III_B and its clones by sequential patient sera. Sequential serum samples obtained from a single patient at the specified intervals were used to investigate the time course of NA against HTLV-III_B (●) and its clones. Neutralization assays were performed with an inoculum of 20 × TCID₅₀ of the respective viruses, and 50% reduction of virus expression was the end point used. The titers shown represent the inverse geometric mean titers (GMT) of duplicate determinations. ○, HX10; ◆, HXB2; ◇, HXB3.

Table 1. Restricted neutralization of viruses derived from HTLV-III_B by selected patient sera. Inverse geometric mean titers (GMT) and standard errors of paired duplicate determinations on different virus preparations with inocula of four times the 50% infectious dose (4 × TCID₅₀) are shown. Complete inhibition of viral expression at 2 weeks was used as the end point. Sera samples in parentheses share similar patterns of NA. All viruses were grown in H9 lymphoblastoid cells, and supernatants were concentrated 1000-fold by ultracentrifugation. Titrations and neutralization assays were done with an immunofluorescence technique to detect HIV-1 p24 expression (1). A 14- to 16-day incubation time was used for both neutralization assays and calculation of TCID₅₀ from eight parallel dilutions.

Sera	Virus neutralization (inverse GMT) for virus			
	HTLV-III _B	HX10	HXB2	HXB3
HS-06 (HS-15, -20)	360 ± 240	430 ± 290	510 ± 320	4000 ± 0
HS-12 (HS-07, -26)	180 ± 150	1600 ± 730	64 ± 0	64 ± 60
HS-23 (HS-22)	23 ± 16	130 ± 100	1000 ± 600	510 ± 390
HS-01 (HS-10, -13, -16)	1 ± 2	11 ± 8	16 ± 0	2000 ± 800
HS-08 (HS-04, -05, -18, -21)	1 ± 0	2 ± 1	4 ± 0	2 ± 1

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