Table 3. Dose-response for compound **XVII** against JM cells infected with HIV-1 (GB8).

XVII (n*M*)	Mean syncytial count	p24 Antigen (ng/ml)
3	25 (19%)	0.88 (2.8%)
1	50 (31%)	1.56 (5.7%)
0.3	71 (53%)	2.65 (9.8%)
0.1	137 (102%)	16.0 (54%)
0.0*	134 (100%)	27.0 (100%)

*Drug free.

a similar enhancement in HIV proteinase inhibition. In contrast to AZT and other inhibitors of the reverse transcription process (*24*), proteinase inhibitors are effective against chronically infected cells. The high level of antiviral activity and low cytotoxicity for compound **XVII** provides a high therapeutic index.

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15. Human leukocyte elastase assays made use of enzyme purified from leukocytes of patients with chronic granulocytic leukemia; methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitro-anilide was the substrate at *p*H 8. Bovine cathepsin B (Sigma Chemical Co., Ltd.) was assayed at *p*H 6.0 with benzoylarginine-*p*-nitroanilide as substrate. Collagenase partially purified from human synovial fibroblast culture medium was assayed by the method of B. Johnson-Wint [*Anal. Biochem.* **104**, 174 (1985)].
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Immunobiology and Pathogenesis of Hepatocellular Injury in Hepatitis B Virus Transgenic Mice

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The role of the immune response to hepatitis B virus (HBV)–encoded antigens in the pathogenesis of liver cell injury has not been defined because of the absence of appropriate experimental models. HBV envelope transgenic mice were used to show that HBV-encoded antigens are expressed at the hepatocyte surface in a form recognizable by major histocompatibility complex (MHC) class I–restricted, $CD8^+$ cytotoxic T lymphocytes specific for a dominant T cell epitope within the major envelope polypeptide and by envelope-specific antibodies. Both interactions led to the death of the hepatocyte in vivo, providing direct evidence that hepatocellular injury in human HBV infection may also be immunologically mediated.

THE HBV IS AN ENVELOPED, CIRCULAR, double-stranded DNA virus that causes acute and chronic liver disease and hepatocellular carcinoma (*1*). The mechanisms responsible for HBV-induced hepatocellular injury are not well understood (*2*). Although HBV-specific T cells are present in the peripheral blood and intrahepatic lymphocyte populations in HBV vaccine recipients (*3, 4*) and patients with chronic hepatitis (*5*), there is no definitive evidence that these responses are involved in the destruction of infected hepatocytes in this disease. The narrow host range of HBV and its nontransmissibility in routine cell culture systems have prevented the use of conventional approaches to this question. The minimal model for T cell–mediated liver cell injury requires hepatocellular synthesis of HBV gene products; the appropriate processing, association, and surface expression of these products with major histocompatibility determinants; the induction and expansion of a cellular immune response specific to MHC-associated HBV peptides; and the susceptibility of the primary hepatocyte to T cell–mediated cytolysis.

We developed an HBV transgenic mouse model system to analyze the immunopathogenesis of HBV-induced liver disease (*6*). The HBV (subtype ayw) envelope region that contained the preS(1), preS(2), and HBs antigens (HBsAg) was ligated to mouse albumin regulatory sequences and introduced into unicellular inbred (B10.D2) mouse embryos (*7*). Transgenic mouse lineage 107-5 [official designation Tg (Alb-1, HBV) Bri66] expresses noncytotoxic amounts of the HBV large and major envelope polypeptides in the hepatocytes (*7*). This lineage does not develop spontaneous liver disease and is immunologically tolerant to the preS and HBs antigens (*7*). Donor B10.D2 mice were immunized with a recombinant vaccinia virus (vHBs4) that contained the coding region for the HBV major envelope polypeptide. Transgenic recipients of primed spleen cells from these

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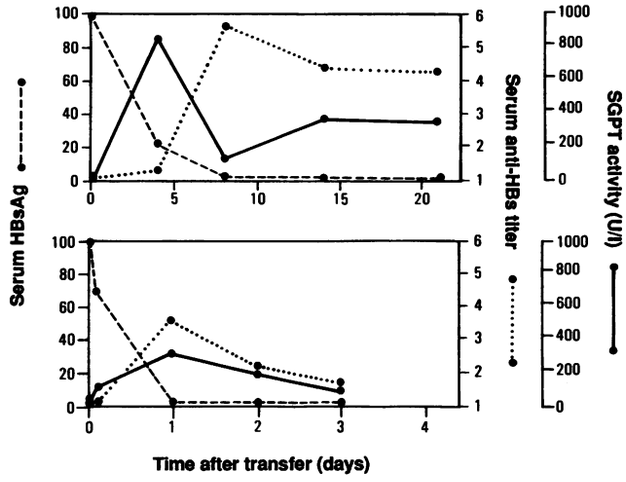

Fig. 1. (**Top**) Nontransgenic, syngeneic (B10.D2) mice were infected four times at biweekly or monthly intervals with 1×10^8 plaque-forming units (PFUs) of vHBs4 that expresses the HBV (subtype adw) major envelope polypeptide that contains HBsAg (*13*) before adoptive transfer of 1×10^8 spleen cells into syngeneic transgenic (107-5) and nontransgenic recipients. Anti-HBs titer in the serum of donor mice at the time of spleen cell harvest was 1×10^{-5} (AUSAB, serial end-point dilution analysis). All recipients were bled at the indicated intervals and the serum was analyzed for SGPT activity (*22*), HBsAg (AUSRIA-II, percentage before transfer), and anti-HBs ($1/\log_{10}$ of the antibody titer). Before transfer, SGPT levels were always less than 100 units per liter. (**Bottom**) Serum (300 μl) containing antibody to HBsAg (titer, 1×10^{-5}) derived from the vHBs4-primed spleen cell donors shown in top was injected intraperitoneally into transgenic recipients. Experiments were performed in triplicate; representative results in transgenic recipients TM289 and TM212 are shown in the top and bottom, respectively. Comparable changes were not observed in nontransgenic controls (*8*).

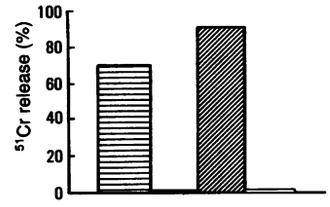

Fig. 3. Antigenic specificity of HBV envelope–specific CTL. A 4-hour assay was performed with ^{51}Cr-labeled target cells as described (*23*). Targets were SV40-transformed transgenic (horizontal bars) and nontransgenic (solid bar) hepatocyte cell lines and P815 (H-2^d) cells transfected (diagonal bars) or not transfected (open bar) with a plasmid vector pMAMneo [Clontech Laboratories and (*24*), (P815-preS1)] containing the HBV (subtype ayw)–envelope fragment spanning nucleotides 2836 to 1407. This fragment directs the synthesis of the HBV large envelope polypeptide (*25*). Effector target cell ratio was 10:1. All target cells were incubated with OH2 culture supernatant (10%) for 24 hours before analysis. Experiments were performed in duplicate. Standard deviations were less than 5.3% of the reported value.

mice displayed biochemical evidence of liver cell injury (Fig. 1, top). The hepatocyte injury was caused by an immune response to the HBV-encoded antigens, because there was no injury detectable in transgenic recipients of spleen cells from donors hyperimmunized with a wild-type vaccinia virus, nor in nontransgenic recipients of vHBs4-primed spleen cells (*8*). The importance of the immune response in this process is confirmed by the observation that the severity and time of onset of liver cell injury in the transgenic recipients was proportional to the degree of sensitization of the primed spleen cell donors (*8*).

Liver cell injury displayed biphasic kinetics after transfer of spleen cells from hyperimmunized donors. The early phase of injury (days 3 to 7) corresponded with the rapid disappearance of HBsAg from the serum and the appearance of free antibodies to HBs (anti-HBs) (Fig. 1, top), suggesting that early injury may be mediated by antibody. The involvement of antibody was confirmed by the passive transfer of high-titer anti-HBs antiserum into transgenic recipients. This induced the clearance of HBsAg from the serum in less than 24 hours, concomitant with the appearance of free circulating anti-HBs and the elevation of serum glutamic-pyruvic transaminase (SGPT) activity with kinetics compatible with immune complex–mediated disease (Fig. 1, bottom). Thus, an antibody-defined target antigen was present on the hepatocyte surface membrane.

The late phase (days 14 to 21) of injury after transfer of vHBs4-primed spleen cells (Fig. 1, top) occurred long after the clearance of HBsAg from the circulation. The delayed onset of injury was also seen after the transfer of spleen cells from mice with low anti-HBs titers. This late phase of liver cell injury was characterized histologically by widespread focal hepatocellular necrosis and a lymphomononuclear cell infiltrate with associated lymphoid infiltration in portal areas (Fig. 2), thus resembling the histological picture of chronic hepatitis in man (*9*).

Because of these features, we focused on the potential role of the cellular immune response in the induction of delayed onset liver cell injury in this model. Lymphocyte subsets were prepared from primed spleen cells by separation on nylon wool followed by depletion with specific antibody plus complement (Table 1) (*10*) and they were injected intraperitoneally into transgenic mice and nontransgenic littermates. Liver cell injury was mediated only by the Lyt 2^+ (CD8) T cell. We established a polyclonal HBsAg-specific, MHC class–restricted cytotoxic T lymphocyte (CTL) line from primed spleen cells by repetitive in vitro stimulation with syngeneic, SV40-transformed transgenic hepatocytes as described (*11*). The

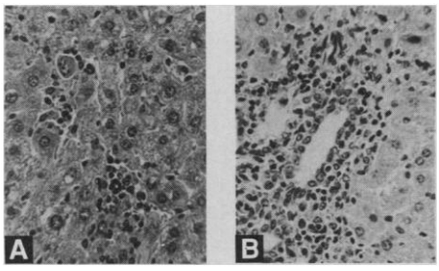

Fig. 2. Histological evidence of liver cell injury and lymphocytic infiltration 2 weeks after adoptive transfer of 1×10^8 vHBs4-primed spleen cells into 107-5 transgenic mice. (**A**) Focal hepatocellular necrosis and parenchymal lymphoid infiltrate are evident. (**B**) Infiltration of lymphomononuclear cells in the portal tract is shown, with destruction of some marginal hepatocytes (piecemeal necrosis). Cells were stained with hematoxylin and eosin; original magnification ×100.

Table 1. Liver injury after adoptive transfer of vKC1-primed lymphocyte subsets. SGPT activity was measured 2 weeks after adoptive transfer of 1×10^7 lymphocytes and results represent the mean (±SEM) SGPT levels of three mice in each group. T and B cells were separated from the total spleen cell population by nylon wool adherence (*21*) and negative selection with subset-specific antibodies plus complement (*10*). Purity of lymphocyte fractions was assessed by flow cytometry with fluorescein-conjugated MAbs to Thy-1.2, L3T4, and Lyt 2 (Becton Dickinson) and with rabbit antibodies to mouse immunoglobulin (Ig) (Zymed).

Subset	Cell surface markers (%)				Recipients (SGPT U/l)	
	Thy-1	Ly 2^+	L3T4	Ig	Transgenic	Normal
T (Thy-1^+)	97.4			4.6	173 ± 21	51 ± 2
CTL (Lyt 2^+)	97.5	87.8	2.9	5.0	155 ± 18	44 ± 1
Th (L3T4^+)	95.1	1.6	68.4		60 ± 2	26 ± 1
B (Ig^+)	0.2			90.4	43 ± 9	28 ± 4

CTL line killed SV40-transformed transgenic hepatocytes and a stably transfected, MHC identical (H-2^d), mouse P815 mastocytoma target cell line that expressed the entire HBV envelope region [P815-preS(1)], but not nontransgenic hepatocytes nor nontransfected control P815 cells (Fig. 3). Cytotoxicity was enhanced when transgenic hepatocyte target cells were treated with gamma-interferon, presumably because of enhanced expression of MHC by the hepatocytes (*11*), which normally express these determinants at very low levels (*12*).

To define the antigenic specificity of the CTL line, P815 target cells were infected with a panel of three recombinant vaccinia viruses that express the major (HBsAg), middle [preS(2) and HBsAg], and large [preS(1), preS(2), and HBsAg] envelope polypeptides (*13–15*). Results were compared with the killing of the stably transfected P815-preS(1) cell line that expresses the large envelope polypeptide. Since all HBV envelope targets were equally susceptible to the cytolytic activity of the CTL line (*16*), the dominant specificity appeared limited to the common carboxy-terminal residues that specify HBsAg.

To determine the fine specificity of the CTL response, we incubated P815 cells with a panel of synthetic peptides that encompassed almost the entire preS and HBsAg sequence. The CTL-killed P815 cells that had been incubated with one of the HBs peptides (HBs2), which corresponds to amino acids 21 to 40 of the major envelope polypeptide (Fig. 4), indicate that this sequence represents an important CTL epitope in the context of the H-2^d haplotype. Eighteen clones were established from the CTL line by limiting dilution at one cell per well (*16, 17*). The antigenic fine specificity of five of these clones was studied and all revealed the same fine specificity ($HBsAg_{21-40}$) as the parental CTL line, suggesting that it might have been monoclonal.

Cytotoxic activity was MHC class I–restricted. Only HBsAg-positive, MHC-identical (H-2^d) P815 target cells were killed by the CTLs, whereas similarly treated HBsAg-positive, allogeneic (H-2^b) mouse thymoma cells (EL-4; ATCC) were not killed (Fig. 5). The MHC class I–restriction element is probably located in the D region, since cytotoxicity was significantly blocked by a monoclonal antibody (MAb) to H-$2D^d$ (*11*) but not by MAb to H-$2K^d$ (*11, 16*). MHC class II elements were not involved since they are not expressed by the P815 target cells (*18*). The CTL line displayed a $CD5^+$ (100%), $CD8^+$ (91.7%), and $CD4^-$ (0.2%) cell surface phenotype by fluorescent flow cytometry analysis (*16*). The $HBsAg_{21-40}$-specific cytolytic activity, therefore, was mediated by a classical, MHC class I (H-$2D^d$)–restricted cytotoxic T lymphocyte.

To test the cytolytic capacity of the $HBsAg_{21-40}$-specific CTL in vivo, we transferred the parental line and a derivative $HBsAg_{21-40}$-specific CTL clone into syngeneic transgenic mice (lineage 107-5) and into their nontransgenic (B10.D2) littermates. Allogeneic (H-2^b) transgenic mice (lineage 50-4) [previously designated Tg (Alb-1, HBV) Bri44] containing the same transgene (*7, 19*) were used as controls. Liver cell injury was detected in syngeneic transgenic mice less than 5 days after transfer of the parental line (Fig. 6), and a derivative clone was detected that displayed the same kinetics and magnitude of injury (*16*), further suggesting the clonal nature of the parental cell line. Injury was not detected after transfer of the same CTLs to syngeneic nontransgenic mice (Fig. 6) or allogeneic transgenic mice (*16*). These data indicate that $HBsAg_{21-40}$-specific, MHC class I–restricted, $CD8^+$ CTLs are functional in vivo and that endogenously synthesized HBV envelope antigens are expressed at the cell surface in a form that is recognized by such CTLs.

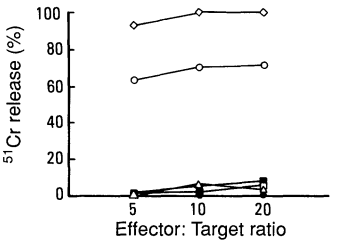

Fig. 5. MHC restriction of HBsAg-specific CTLs. Cytotoxicity was assessed with MHC-identical (P815, H-2^d) and allogeneic (EL4, H-2^b) targets, either transfected with pMAMneo-HBV env [P815-preS(1), open circle; EL4-preS(1), triangle] or incubated with the HBs2 synthetic peptide (P815, diamond; EL4, solid square). Untreated parental cell lines (P815, solid circle; EL4, open square) were used as negative controls (4-hour ^{51}Cr-release assay was as described in the legend to Fig. 3). Effector:target cell ratios were as shown, studies were performed in duplicate, and standard deviations were less than 4.9% of the reported value. The data shown are representative of two independent experiments.

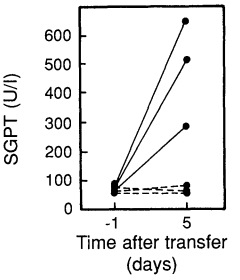

Fig. 6. Functional properties of $HBsAg_{21-40}$-specific CTL line in vivo in HBV transgenic mice. $HBsAg_{21-40}$-specific CTLs (1×10^7) were injected intraperitoneally into groups of three syngeneic transgenic mice (solid line) and three nontransgenic littermates (broken line), and SGPT levels were measured 5 days later. Each point represents the SGPT activity in a single animal. The data presented are representative of two independent experiments.

Our data show that antigen-specific immune effector mechanisms can destroy HBV-positive hepatocytes in vitro and in vivo and demonstrate the experimental induction of classical $CD8^+$, MHC class I–restricted, HBV-specific CTLs in response to, and specific for, endogenously synthesized HBV protein that was expressed in an immunologically recognizable manner at the hepatocyte surface in vivo. Together these observations establish the minimal requirements for a pathophysiologically relevant CTL response in vivo. Antiviral antibodies may also be able to destroy infected hepatocytes, a concept that has been, heretofore, considered unlikely (*20*). Since the experimental design favored the induction of an HBsAg group–specific immune response, further studies designed to examine the pathogenetic potential of antibody and CTLs

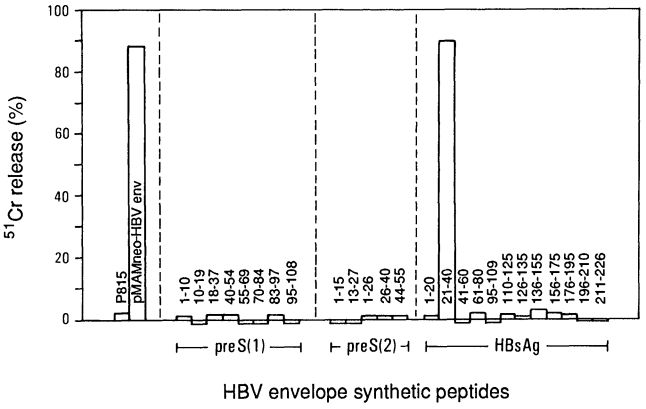

Fig. 4. Antigenic fine specificity of HBsAg-specific CTL. Cytotoxicity analysis was performed with MHC-identical P815 (H-2^d) cell targets incubated during the 4-hour assay period with a panel of synthetic peptides (20 nucleotide oligomers at 10 μg/ml) corresponding to the HBV-envelope region (that is, preS and HBs) prepared by Multiple Peptide Systems. Cytotoxicity results were compared with the killing of P815 cells that had been transfected with pMAMneo-HBV envelope or with nontransfected P815 cells as positive and negative controls. Residues included in each peptide are indicated above each bar. The composition of HBs2 peptide is LLTRILTIPQSLDSWWTSLN. The effector:target cell ratio was 5:1, experiments were performed in duplicate, and standard deviations were less than 1.5% of the reported value. The data shown are representative of three independent experiments.

specific for the remaining HBV-encoded antigens in multiple MHC backgrounds are needed to fully understand the relative ability of the response to each HBV antigen to cause liver cell injury in viral hepatitis. The current studies in the HBV transgenic mouse system also represent a model for the analysis of the immunobiology and pathogenesis of other viral diseases for which alternative experimental systems do not exist.

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10. Spleens were harvested from mice 1 week after the third immunization with 1×10^8 plaque-forming units (PFU) of vaccinia recombinant vKC1 at which time their antibody titer was 3.2×10^{-3}. The recombinant vaccinia virus vKC1 expresses the middle and major envelope polypeptides of HBV (subgroup adw) containing the preS(2) and HBs antigens (*14*). This construct is more immunogenic than vHBs4 with respect to the induction of HBsAg-specific CTLs (*16*). Nylon wool–adherent cells were incubated with MAb AT.83 (specific for Thy-1) plus rabbit complement (Cedarlane) to eliminate T cells [D. P. Dialynas *et al.*, *Immunol. Rev.* **74**, 645 (1983)]. Nylon wool nonadherent cells were further purified by incubation with MAb J11D (ATCC) plus rabbit complement to eliminate contaminating B cells. CTLs and helper T cells (Th) were further purified by the addition of MAbs RL172.4 [L3T4-specific; R. Ceredig, J. W. Lowenthal, M. Nabholz, H. R. MacDonald, *Nature* **314**, 98 (1985)] and 3.155 [Lyt 2-specific; D. P. Dialynas, above] plus complement, respectively.
Nontransgenic, syngeneic (B10D.2) mice were immunized twice with 1×10^8 PFU of vKC1. Primed spleen cells were cultured with irradiated (3000 rad) SV40-transformed transgenic hepatocytes (1×10^5 per well) and irradiated (3000 rad) syngeneic spleen cells (4×10^5 per well) in EHAA media (Gibco) that contained recombinant interleukin 4 (IL-4) [2 U/ml, a gift from H. Spiegelberg; L. Smith, *Nature* **326**, 798 (1987)] and IL-6 [32 U/ml, Boehringer Mannheim; M. Okada *et al.*, *J. Immunol.* **141**, 1543 (1988)] in 96-well flat-bottomed plates. Newborn hepatocytes from transgenic and nontransgenic pups were immortalized by transfection with SV40 DNA and were cultured [C. Woodworth, T. Secott, H. C. Isom, *Cancer Res.* **46**, 4018 (1986)]. The transgenic hepatocyte cell line secreted immunoreactive HBsAg into the culture supernatant. The transgenic hepatocytes were incubated with CTL line OH2 supernatant (10%) that contained gamma-interferon [O. Kanagawa, *J. Immunol. Methods* **110**, 169 (1988)] for 24 hours before incubation with primed spleen cells. Expression of MHC class I increased ten times, as observed by flow cytometric analysis with MAbs 34-5-8S and SF1-1.1.1 (ATCC), which are specific for H-2D^d and H-2K^d, respectively. Spleen cell cultures were restimulated weekly with irradiated SV40-transformed liver cells and feeder cells and with EL4 culture supernatant (5%) as a source of IL-2.
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HIV-1 Coat Protein Neurotoxicity Prevented by Calcium Channel Antagonists

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Coat protein gp120 from the human immunodeficiency virus type–1 (HIV-1) increased intracellular free calcium and injured rodent retinal ganglion cells and hippocampal neurons in culture. Highly purified recombinant gp120 envelope protein produced these effects in a dose-dependent fashion at picomolar concentrations. Immunoprecipitation with antibody to gp120, but not with control immunoglobulin-containing serum, depleted solutions of the viral envelope protein and also prevented both the rise in intracellular calcium and neuronal toxicity. The gp120-induced increase in intracellular calcium was abrogated by transiently lowering extracellular calcium or by adding the dihydropyridine calcium channel antagonist nimodipine (100 n*M*). Calcium channel antagonists also prevented gp120-induced neuronal injury. In addition, intracellular stores appeared to contribute substantially to the increase in calcium elicited by gp120. Since increases in intracellular calcium have been associated with neurotoxicity, it is possible that an injurious effect of gp120 on neurons might be related to this mechanism and that treatment with calcium channel antagonists may prove useful in mitigating HIV-1–related neuronal injury.

BOTH NATIVE AND RECOMBINANT viral envelope protein gp120 of HIV-1 have been found, even in the absence of infectivity, to produce neuronal cell injury in the mammalian central nervous system, specifically in cultures of hippocampal (*1*) and retinal ganglion cell neurons (*2*). This finding may account at least in part for the neurological manifestations of dementia (*3*) and blindness (*4*) encountered in acquired immunodeficiency syndrome (AIDS). An increase in intracellular neuronal Ca^{2+} is associated with and apparently responsible for several forms of neurotoxicity, including that mediated by excitatory amino acids binding at the *N*-methyl-D-aspartate receptor (*5, 6*). Thus, a reasonable mechanism for gp120 neurotoxicity might be postulated to involve an increase in intracellular Ca^{2+}. Furthermore, since gp120 had been reported to increase intracellular Ca^{2+} in T lymphocytes (*7*), we decided to examine the effect of this viral envelope protein on Ca^{2+} in mammalian central neurons.

The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured in postnatal rat retinal ganglion cells (*8*) by digital imaging microscopy with the Ca^{2+}-sensitive fluorescent dye fura 2 (*9*). Application of 200 p*M* of highly purified gp120 from a recombinant source (*10*) produced a striking increase in $[Ca^{2+}]_i$ (Fig. 1). Compared to control levels ($[Ca^{2+}]_i = 63 \pm 4$ n*M*, mean ± SEM, $n = 42$) obtained before the addition of coat protein, levels increased 33-fold

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