coronary tone, thereby ameliorating the effects of reactive hyperemia (*11, 17*).

The link between alternans and vulnerability is underscored by the finding that alternans coincides with the established timing of the vulnerable period in the cardiac cycle (*21–23*). Superimposition of successive beats indicates that alternation is restricted to the first half of the T wave (Fig. 1, right panels). This relation remained constant in all animals studied under the changing conditions of sympathetic nervous system stimulation or denervation (Fig. 2, A to C).

The precise electrophysiologic basis for the correspondence between alternans and vulnerability remains to be determined. One possibility is that alternans represents increased dispersion of repolarization, which is most marked during the vulnerable period and which is increased by interventions that enhance susceptibility to fibrillation (*22–24*). Smith and Cohen proposed that alternans may be due to the summation of electrical activity of subpopulations of myocardial cells that generate action potentials only on alternate beats (*25*). Others have ascribed alternans to the action potential morphology of the individual myocardial cells (*15, 26*). Priori and co-workers observed that during coronary artery reperfusion 2:1 block of early afterdepolarization (EAD) conduction occurred simultaneously with the onset of T wave alternans in the intracavitary ECG (*27*). El-Sherif and coworkers reported summation of repolarization activity due to EADs in animals treated with the inotropic agent anthopleurin A (*28*).

From a biophysical perspective, T wave alternans may represent a prechaotic state because bifurcative behavior is the hallmark of chaos (*29–33*). Recent studies by Chialvo and others indicate that myocardial cells can exhibit chaotic dynamics (*29, 30*). To establish with certainty that T wave alternans represents prechaotic behavior requires demonstration of multupling just before fibrillation occurs. Although T wave multupling has been observed during infusion of high concentrations of norepinephrine (*31*), the progression to lethal arrhythmia remains to be demonstrated. This may prove elusive because higher order bifurcations represent extremely unstable, evanescent states.

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Recombinant Virus Vaccine–Induced SIV-Specific CD8$^+$ Cytotoxic T Lymphocytes

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Evidence indicates that cytotoxic T lymphocytes (CTLs) may be important in containing the spread of the human immunodeficiency virus (HIV) in the infected host. Although the use of recombinant viruses has been proposed as an approach to elicit protective immunity against HIV, the ability of recombinant viral constructs to elicit CD8$^+$ CTL responses in higher primates has never been demonstrated. A live recombinant virus, vaccinia–simian immunodeficiency virus of macaques (SIV_{mac}), was used to determine whether such a genetically restricted, T lymphocyte–mediated antiviral response could be generated in a primate. Vaccinia-SIV_{mac} vaccination elicited an SIV_{mac} Gag-specific, CD8$^+$ CTL response in rhesus monkeys. These CTLs recognized a peptide fragment that spans residues 171 to 195 of the Gag protein. The rhesus monkey major histocompatibility complex (MHC) class I gene product restricting this CTL response was defined. Both the vaccinated and SIV_{mac}-infected monkeys that shared this MHC class I gene product developed CTLs with the same Gag epitope specificity. These findings support the use of recombinant virus vaccines for the prevention of HIV infections in humans.

LIVE, ATTENUATED VIRUSES HAVE been used when possible in vaccination, as they elicit longer lasting immunity than that achieved by inactivated virus or protein immunization (*1*). However, in view of the propensity of HIV to undergo rapid mutations (*2*), such an approach for the prevention of acquired immunodeficiency syndrome (AIDS) does not seem feasible. The use of recombinant viruses may result in a level of immunity comparable to that attained in live viral infections (*3*). Thus, HIV genes encoding proteins crucial for eliciting immunity might be incorporated into an established, tolerated viral vector. Although recombinant viruses

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have been shown to elicit humoral immune responses (*3*), they have not been shown to be capable of eliciting a $CD8^+$ CTL response in outbred species. In view of evidence of the importance of effector T lymphocyte immunity in containing an HIV infection (*4–9*), a successful recombinant HIV vaccine should elicit specific CTLs, as well as humoral immune responses.

The SIV-macaque model is proving useful in research efforts to develop an HIV vaccine. SIV_{mac} has extensive sequence similarity to HIV-1 and HIV-2 (*10, 11*) and, like these human viruses, is tropic for $CD4^+$ lymphocytes and macrophages (*12, 13*). After infection with SIV_{mac}, rhesus monkeys develop lymphadenopathy and immunologic abnormalities that include a decline in circulating $CD4^+$ lymphocytes. Infected monkeys eventually die from a spectrum of opportunistic infections and tumors similar to those seen in HIV-infected humans (*14*). SIV_{mac}-infected macaques are, therefore, a useful model for studying HIV infection in humans. Success in preventing SIV infection in macaque monkeys with vaccine preparations made of inactivated virus has led to an optimistic outlook on the prospects of developing an effective HIV vaccine (*15, 16*).

We have initiated studies to elucidate the SIV_{mac}-specific CTL responses in virus-infected rhesus monkeys (*17–20*). We concentrate here on the response to the Gag protein because *gag* is conserved among HIV isolates and because the effector response to this protein appears to be of limited complexity. The effector T lymphocyte response to the SIV_{mac} Gag protein in peripheral blood lymphocytes (PBLs) in a number of rhesus monkeys is $CD8^+$ and restricted by a single major histocompatibility complex (MHC) class I gene product. Synthetic peptides were used to map the epitope recognized by these effector T cells to a single 25–amino acid fragment (peptide 11) of Gag, which spans residues 171 to 195 of the protein. Cloning and sequencing has shown that the monkey MHC class I molecule that binds and presents this peptide fragment to CTL is a human leukocyte antigen–A homolog called *Macaca mulatta* (Mamu)–A1 (*19*). Thus, any SIV_{mac}-infected rhesus monkey bearing Mamu-A1 develops a $CD8^+$ Gag-specific CTL response with a peptide 11 specificity.

To detect HIV- or SIV-specific CTLs, researchers use vaccinia–recombinant viruses to express HIV proteins in target cell populations (*5, 6, 17*). This approach cannot be used for detecting SIV_{mac}-specific CTLs in PBLs of vaccinia-SIV_{mac}–vaccinated monkeys because such animals would also be expected to generate vaccinia-specific CTLs that would obscure SIV_{mac}-specific responses. However, we reasoned that if

Table 1. A 25–amino acid fragment of the Gag protein of SIV_{mac} sensitizes target cells for lysis by PBLs of vaccinia-SIV_{mac}–infected monkeys. We generated rhesus monkey B lymphoblastoid cell lines for use as target cells by incubating PBLs with *Herpesvirus papio*–containing culture supernatant. The SIV_{mac} Gag peptides used were 25 amino acids in length, each consecutive peptide overlapping the preceding one by 8 amino acids. Peptide 11 contains the sequence: VPGFQALSEGCTPYDINQMLNCVGD (*32*) [positions 171 to 195 of the sequence of Franchini *et al.* (*11*)]. Target cells were incubated with peptide 9, 10, or 11 at a final concentration of 50 μg/ml for 16 hours at 37°C. Effector cells were generated from Ficoll-diatrizoate density centrifugation–isolated PBLs that were obtained 25 days after vaccinia-SIV_{mac} or vaccinia-control vaccination. Cells were cultured for 3 days at 10^6 cells per milliliter with concanavalin A (5 μg/ml) (Sigma). We washed the cells and then maintained them another 3 days in medium supplemented with human recombinant interleukin-2 (20 U/ml) (provided by Hoffmann–La Roche, Nutley, New Jersey). Target cells (T, 10^4) were labeled with ^{51}Cr and incubated for 5 hours with varying numbers of effector (E) cells. Spontaneous release of target cells varied from 15 to 25%. Percent specific release for each listed peptide was calculated by the equation [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100.

Effector cells	Infection	E:T ratio	Specific release (%)		
			9	10	11
Mm 352-88	Vac-SIV_{mac}	100:1	5	8	21
		50:1	4	7	15
		25:1	2	4	7
		12.5:1	2	1	8
Mm 173-84	Vac-SIV_{mac}	100:1	12	10	27
		50:1	9	6	22
		25:1	4	9	17
		12.5:1	8	5	14
Mn 348-88	Vac-control	100:1	10	8	12
		50:1	12	8	14
		25:1	3	5	6
		12.5:1	0	2	9
Mm 297-88	Vac-control	100:1	11	11	11
		50:1	1	4	9
		25:1	0	0	9
		12.5:1	0	0	4
Mm 152-86	SIV_{mac}	100:1	12	8	59
		50:1	10	9	50
		25:1	6	7	40
		12.5:1	4	4	33

Table 2. The SIV_{mac} Gag peptide 11–specific effector cells in the vaccinia-SIV_{mac}–infected monkeys are $CD8^+$. PBLs were incubated with monoclonal antibodies to CD4 [19Thy5D7; (*33*)] or CD8 [7Pt3F9; (*33*)] at 1:500 dilutions for 40 min at 4°C. We washed the cells and then incubated them with goat anti-mouse immunoglobulin–coated Dynabeads (Dynal) (10^6 cells per 100 μl of Dynabeads) for 40 min at 4°C. We depleted the culture of $CD4^+$ or $CD8^+$ lymphocytes in two cycles with a magnetic particle concentrator. Monoclonal antibody staining and flow cytometric analyses were performed on the resulting cell populations. The immunophenotypes of the effector cells were as follows: $CD4^+$-depleted Mm 352-88 cells were <1% $CD4^+$, 89% $CD8^+$; $CD4^+$-depleted Mm 173-84 cells were 1% $CD4^+$, 95% $CD8^+$; $CD4^+$-depleted Mm 152-86 cells were <1% $CD4^+$, 96% $CD8^+$; $CD8^+$-depleted Mm 352-88 cells were 88% $CD4^+$, <1% $CD8^+$; $CD8^+$-depleted Mm 173-84 cells were 97% $CD4^+$, <1% $CD8^+$; and $CD8^+$-depleted Mm 152-86 cells were 82% $CD4^+$, 1% $CD8^+$. The peptide-specific ^{51}Cr release assay was performed as described in Table 1. The percent specific peptide 11 release was calculated as the percent peptide 11 release − percent peptide 10 release.

Effector cells	Infection	E:T ratio	Peptide 11 specific release (%)	
			CD4-depleted	CD8-depleted
Mm 352-88	Vac-SIV_{mac}	100:1	30	3
		50:1	21	3
		25:1	17	3
		12.5:1	10	0
Mm 173-84	Vac-SIV_{mac}	100:1	25	0
		50:1	21	2
		25:1	19	1
		12.5:1	20	0
Mm 152-86	SIV_{mac}	100:1	19	0
		50:1	15	0
		25:1	12	0
		12.5:1	8	0

CTLs could be generated by vaccinia-SIV_{mac} vaccination in Mamu-$A1^+$ rhesus monkeys, we should be able to detect the lysis of SIV_{mac} Gag peptide 11–treated target cells. Therefore, four healthy Mamu-$A1^+$ rhesus monkeys were selected by screening their PBLs for Mamu-A1 expression using radioimmunoprecipitation and one-dimensional isoelectric focusing (*21*).

We established B lymphoblastoid cell lines (B-LCL) from the PBLs of these animals. These B-LCL, after infection with vaccinia-SIV_{mac} *gag* or incubation with SIV_{mac} Gag peptide 11, were susceptible to lysis by the CTLs of a Mamu-$A1^+$, SIV_{mac}-infected monkey (*21*). Before vaccination of the selected animals, their PBLs did not lyse autologous target cells infected only with vaccinia-SIV_{mac} *gag* or treated with the SIV_{mac} Gag peptide 11. Two of the monkeys were then vaccinated intradermally with 6×10^8 plaque-forming units (pfu) of a multivalent vaccinia construct containing both SIV_{mac} *env* and SIV_{mac} *gag-pol* (vAbT 386-6-1). We used a multivalent vaccine to facilitate the evaluation of various immune responses to the SIV_{mac} structural proteins.

The other two monkeys received a control vaccination with 6×10^8 pfu of a vaccinia construct containing the equine herpes virus (EHV) *gH* gene (vAbT 249-3-1). The New York City Board of Health strain of vaccinia was used as the parental virus for the construction of the recombinant viruses. The SIV_{mac} and EHV genes were inserted into the Hind III M fragment by means of a host range selection vector and, therefore, retained an active thymidine kinase gene. Expression of the genes was controlled by vaccinia promoters active both early and late in the vaccinia replication cycle. Monkey cells infected with vAbT 386-6-1 expressed all appropriate SIV_{mac} gene products, including the envelope glycoproteins, the correctly processed core polypeptides, and reverse transcriptase (*22*).

Monkeys vaccinated with SIV_{mac}-specific constructs, but not those vaccinated with control preparations, developed antibody responses to SIV_{mac} assessed by indirect immunofluorescence on SIV_{mac}-infected H9 cells; peak titers of 1:160 were reached 60 days after vaccination. Moreover, PBL proliferative responses to the recombinant protein SIV_{mac} gp140 demonstrated stimulation indices of approximately 3 on day 18 after virus inoculation in the vaccinia-SIV_{mac}, but not in the vaccinia-control immunized monkeys (*23*). Thus, the vaccinia-SIV_{mac} immunized monkeys developed both humoral and T helper cell SIV_{mac}-specific immunity.

PBLs of the vaccinia-SIV_{mac} vaccinated animals also mediated lysis of SIV_{mac} Gag peptide 11–treated target cells but did not mediate lysis of targets incubated with other SIV_{mac} Gag peptides (Table 1). PBLs of the vaccinia-control vaccinated monkeys did not mediate any significant amount of lysis of SIV_{mac} peptide 11–pulsed target cells. The peptide 11–specific target cell lysis was mediated by $CD8^+$ PBLs because $CD8^+$ lymphocyte–depleted PBLs did not lyse these targets (Table 2). Moreover, these vaccinia-SIV_{mac}–induced effector cells were MHC class I–restricted in their target cell lysis (Table 3). These effector lymphocytes lysed peptide 11–treated target cells if they were autologous or Mamu-$A1^+$ allogeneic, but not Mamu-$A1^-$ allogeneic. This peptide 11–specific CTL response was only detectable in concanavalin A–activated, interleukin-2–expanded PBLs; it was observed for 4 months after vaccination.

Recombinant vaccinia viruses elicit $CD8^+$ CTL responses to influenza, herpes simplex, murine leukemia, and human immunodeficiency viruses in mouse model systems (*24–27*). Immunization protocols that include recombinant vaccinia viruses expressing the HIV envelope glycoprotein and recombinant protein gp160 as soluble proteins induce $CD4^+$ effector T cells in chimpanzees and humans (*28–30*). However, a role for $CD4^+$ effector T lymphocytes in host defenses against viral infections remains to be proven. $CD8^+$, MHC class I–restricted CTLs are important in containing the spread of virus in the infected host (*31*). Our experiments demonstrate that a $CD8^+$ CTL response to a conserved structural protein of SIV_{mac} can be elicited in a higher primate by means of a recombinant viruses vector system. These findings support the feasibility of the use of recombinant viruses in vaccines for the prevention of HIV infection in humans.

Table 3. Lysis of SIV_{mac} Gag peptide 11–sensitized target cells by PBLs of vaccinia-SIV_{mac}–infected monkeys is MHC class I–restricted. Matched target cells were derived from a monkey that shared the Mamu-A1 allele in common with the effector PBLs; mismatched target cells were Mamu-$A1^-$. Thus, for Mm 352-88 effector cells, matched targets were B-LCL from Mm 173-84 and Mm 244-86. For Mm 173-84 effector cells, matched target cells were from Mm 352-88 and Mm 244-86. For Mm 244-86 effector cells, matched targets were from Mm 173-84 and Mm 352-88. Mismatched target cells for all effector cells were from Mm 174-88 and Mm 246-88. The assay was performed as described in Tables 1 and 2.

Effector cells	Infection	E:T ratio	Peptide 11 specific release (%)				
			Autologous	Matched		Mismatched	
Mm 352-88	Vac-SIV_{mac}	100:1	16	27	15	0	0
		50:1	12	16	15	0	0
		25:1	1	13	8	0	0
		12.5:1	6	12	5	0	2
Mm 173-84	Vac-SIV_{mac}	100:1	12	10	12	2	0
		50:1	11	8	12	0	0
		25:1	3	3	4	2	3
		12.5:1	4	5	5	4	0
Mm 297-88	Vac-control	100:1	2				
		50:1	1				
		25:1	2				
		12.5:1	0				
Mm 348-88	Vac-control	100:1	4				
		50:1	1				
		25:1	0				
		12.5:1	0				
Mm 244-86	SIV_{mac}	100:1	47	49	39	0	0
		50:1	37	35	26	0	0
		25:1	28	25	15	0	2
		12.5:1	16	12	6	0	0

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32. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M. Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Calcium as a Coagonist of Inositol 1,4,5-Trisphosphate–Induced Calcium Release

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Inositol 1,4,5-trisphosphate (IP_3)–induced calcium release from intracellular stores is a regulator of cytosolic-free calcium levels. The subsecond kinetics and regulation of IP_3-induced calcium-45 release from synaptosome-derived microsomal vesicles were resolved by rapid superfusion. Extravesicular calcium acted as a coagonist, potentiating the transient IP_3-induced release of calcium-45. Thus, rapid elevation of cytosolic calcium levels may trigger IP_3-induced calcium release in vivo. Extravesicular calcium also produced a more slowly developing, reversible inhibition of IP_3-induced calcium-45 release. Sequential positive and negative feedback regulation by calcium of IP_3-induced calcium release may contribute to transients and oscillations of cytosolic-free calcium in vivo.

A VARIETY OF EXTRACELLULAR SIGnals regulate intracellular processes by triggering increases in cytosolic Ca^{2+} concentration. Such increases are produced by entry of Ca^{2+} across the plasma membrane or release of Ca^{2+} from intracellular stores, or both. IP_3 is a phospholipid metabolite that couples the activation of cell surface receptors to changes in intracellular Ca^{2+} concentration by stimulating Ca^{2+} release from intracellular stores (*1*). An IP_3 receptor from mammalian brain has been purified (*2*), cloned (*3*), and reconstituted in lipid vesicles (*4*), and it appears that the IP_3 binding site and the Ca^{2+} release channel reside in a single protein. Elevation of cytosolic or extravesicular Ca^{2+} (Ca_e^{2+}) inhibits IP_3-induced Ca^{2+} release (*5*). To clarify the relationship between Ca_e^{2+} and the IP_3 receptor, we used a rapid superfusion system (*6*) to study $^{45}Ca^{2+}$ release from microsomal vesicles derived from rat brain synaptosomes, a nerve terminal preparation.

Microsomal vesicles were obtained by hypotonic lysis and discontinuous sucrose gradient centrifugation of rat brain synaptosomes (*7*). This resulted in six fractions, two of which (D and E) released accumulated $^{45}Ca^{2+}$ when exposed to IP_3. Fraction D was more active and was chosen for further characterization.

The vesicles actively accumulated $^{45}Ca^{2+}$ in an adenosine triphosphate (ATP)–dependent manner (*8*), and were retained on filters in a superfusion chamber accessed by three solenoid-driven valves that were operated by computer (*6*). Each valve controlled the delivery of a separate pressurized solution to the chamber. The $^{45}Ca^{2+}$ containing effluent was continuously collected by a high-speed fraction collector. The high flow rate (1 to 2 ml/s) relative to the small dead volume of the superfusion chamber (30 μl) allowed rapid solution changes and precise control of the extravesicular concentrations of IP_3 and Ca^{2+} (*9*), and afforded a time resolution ≤70 ms (*6*). The resulting rapid removal of released $^{45}Ca^{2+}$ isolated the efflux from other processes, such as reuptake, Ca^{2+} buffering, and agonist depletion. Thus, the effects of various agents on release could be readily quantified.

Continuous superfusion of vesicles with IP_3 resulted in a transient release of $^{45}Ca^{2+}$ (Fig. 1A). Vesicles were initially superfused with a buffer containing 100 nM Ca^{2+}, and $^{45}Ca^{2+}$ release was evoked by superfusion with a buffer containing 1 μM IP_3 and 10 μM Ca^{2+}. The release rate (*10*) reached a maximum within 140 ms after introduction of IP_3 and decayed exponentially over the next second (*11*). The IP_3-stimulated release was blocked by heparin (100 μg/ml), which inhibits the binding of IP_3 to its receptor (*2*). There was an additional smaller and kinetically distinct component of $^{45}Ca^{2+}$ release, stimulated by Ca_e^{2+} alone. It was not sensitive to heparin, but was nearly completely blocked by 3 to 5 mM Mg^{2+} (Fig. 1B). At ≤5 mM, Mg^{2+} had no effect on net $^{45}Ca^{2+}$ release evoked by 1 μM IP_3 [calculated as described in (*12*)]. The selective block of the IP_3-induced $^{45}Ca^{2+}$ release by heparin and the Ca_e^{2+}-mediated component of $^{45}Ca^{2+}$ release by Mg^{2+} suggested that each of these processes was mediated by an independent release mechanism and provided pharmacological criteria for distinguishing them (*12*).

The dependence on IP_3 concentration of the maximum observed rate of net $^{45}Ca^{2+}$ release (*12*) (Fig. 2) indicated that IP_3 concentration and $^{45}Ca^{2+}$ release rate were related in a normal hyperbolic manner over the range of 30 nM to 10 μM. The Hill coefficient (n_H) ± SD was 1.0 ± 0.2, and the half-maximal concentration (EC_{50}) was 240 ± 60 nM (Fig. 2, inset). At saturating IP_3 concentrations (10 μM), only 6% of the total accumulated $^{45}Ca^{2+}$ was released during the 2-s stimulus, whereas all of the $^{45}Ca^{2+}$ was discharged by the Ca^{2+} ionophore A23187 (1 μM).

We then explored possible explanations for the transient nature of the IP_3-evoked $^{45}Ca^{2+}$ release. If decay was due to depletion of releasable $^{45}Ca^{2+}$, the total amount of isotope released would have been the same for all IP_3 concentrations, but the rate of decay would have increased at higher agonist concentrations (Fig. 2). In contrast, the amount of $^{45}Ca^{2+}$ released increased as a function of IP_3 concentration (Fig. 2, inset), whereas the time constant for decay of the $^{45}Ca^{2+}$ release was constant for each Ca_e^{2+} concentration (*13*). Gramicidin D (300 nM), a monovalent cation ionophore used to collapse a transmembrane electrostatic gradient that might develop from a net efflux of cations, did not alter the kinetics of $^{45}Ca^{2+}$ release (*14*). These observations suggested that receptor inactivation, rather than depletion of intravesicular $^{45}Ca^{2+}$ or electrostatic forces, accounted for the rapid decay of the $^{45}Ca^{2+}$ release.

The magnitude and the time course of IP_3-induced $^{45}Ca^{2+}$ release were modulated

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