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TECHNICAL COMMENTS

Attenuated Retrovirus Vaccines and AIDS

In their recent report, Timothy W. Baba *et al.* state that a deletion mutant of simian immunodeficiency virus (SIVΔ3), which does not cause disease in adult macaques and has been successfully used as a vaccine against challenge with pathogenic virus (*1*), causes acquired immunodeficiency syndrome (AIDS) in newborn macaques. They ascribe the differential outcome of SIVΔ3 infection of neonatal and adult macaques to several possibilities including the amount of virus replication early after inoculation, the route of virus inoculation, and the developing neonatal immune system. However, their study does not allow separation of these important variables.

We found that high-dose intravenous inoculation of newborn rhesus macaques with molecularly cloned SIVmac239 (the parental virus from which SIVΔ3 was derived) resulted in persistently high amounts of virus in peripheral blood mononuclear cells (PBMC) and plasma (higher than those reported by Baba *et al.* for SIVΔ3). Rhesus newborns infected with SIVmac239 did not experience rapid $CD4^+$ T lymphocyte depletion, and the time course before fatal immunodeficiency developed was consistent with that previously reported for SIVmac239-infected adult macaques (that is, 6 to 24 months) (*2, 3*). Thus, an age-related difference does not explain why rhesus infants inoculated with an attenuated triple-deletion mutant of SIVmac239 appear to experience a more rapid $CD4^+$ T cell depletion and $CD4^+/CD8^+$ T cell ratio inversion than rhesus infants inoculated with the pathogenic parental virus, SIVmac239. We also found that absolute $CD4^+$ T lymphocyte numbers were not a reliable marker of disease progression in infant rhesus macaques because of extreme variability of absolute lymphocyte counts in response to stress (for example, handling). Only absolute $CD4^+$ T cell numbers that are persistently below 500 per microliter reliably suggested $CD4^+$ T lymphocyte depletion in neonatal macaques (*2–4*).

Baba *et al.* hypothesize that the oral route of inoculation may be responsible for increased virulence of SIVΔ3 in newborns. Our observations with five orally and six intravenously inoculated newborn macaques did not demonstrate a more severe course of infection with uncloned pathogenic SIVmac251 for the oral route (*2–4*). With regard to the postulated age-dependence of SIV virulence, we have also compared the time course of infection of the nonpathogenic molecular clone, SIVmac1A11, and SIV/human immunodeficiency virus–1 (HIV-1) envelope chimeric viruses in macaques of different ages: We have no evidence that an SIV strain that is attenuated in older macaques becomes pathogenic when inoculated intravenously or orally into newborn macaques (*2, 5*). Instead, inoculation of fetal and newborn macaques with attenuated SIVmac1A11 proved to be a safe and effective vaccine against challenge with pathogenic uncloned SIVmac later in life (*3*). Finally, our studies indicate that the neonatal immune system was not overwhelmed by attenuated SIV isolates or by a pathogenic SIV clone (*2, 3*).

Caution must be used when assigning the underlying cause of death in SIV-infected macaques to immunodeficiency. For the one SIVΔ3-inoculated macaque that died in their study, the classical hallmarks of simian AIDS (such as the presence of opportunistic infections, encephalopathy, and so on) apparently were not demonstrated by Baba *et al.* Instead, this animal had severe anemia and thrombocytopenia, reportedly a result of peripheral autoimmune destruction of red blood cells and platelets. It is not clear whether this diagnosis of hemolytic anemia was mainly based on a positive direct Coombs test. Many healthy macaques will react positively if human Coombs test reagents are used (*6*). Clinical hemolytic anemia must be confirmed by additional evidence, such as hemoglobinuria, poikilocytosis, the presence of spherocytes, hemolytic or icteric plasma, and increased serum bilirubin and lactate dehydrogenase. The erythroid hyperplasia of the bone marrow, reported by Baba *et al.*, is a finding that we do not see in anemic SIV-infected animals; rather, their bone marrow aspirates reveal a myeloid hyperplasia with the erythroid series being normal or only slightly increased (*7*). Findings in addition to an abundance of megakaryocytes in the bone marrow are needed to support the hypothesis of peripheral platelet destruction. SIV-infected animals often have a megakaryocyte hyperplasia of the bone marrow, but these megakaryocytes have increased cytoplasmic vacuolization, which suggests that the thrombocytopenia is a result of decreased platelet production rather than peripheral platelet destruction (*6*).

Extra care needs to be taken to exclude all other pathogens that can adversely affect the immune system and the health of macaques. Although the animals in the study by Baba *et al.* were polymerase chain reaction–negative (by an assay able to detect approximately one infected cell in 8000 PBMC) and seronegative for simian type D retroviruses, virus isolation is more reliable for diagnosis of this viral infection, but was not reported by Baba *et al.*

Until a more thorough analysis is completed and results of Baba *et al.* are confirmed, it would be premature to dismiss the potential of SIV *nef*-deletion mutants as live-attenuated vaccines.

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The report by Baba *et al.* raises concern that attenuated HIV may not be safe as a vaccine to prevent syndrome AIDS. A major point of the report is that mucosal infection of newborn macaques with a triple gene-deleted preparation of attenuated SIV can result in an AIDS-like condition with a reduction in the CD4 cell count. This observation contrasts with the experience of Desrosiers and his colleagues who observed no ill effects in adult macaques that had been infected with a triple deletion SIV (*1*). Although the differences observed in the two studies might be accounted for by differences in the doses and routes of attenuated SIV administered, as noted by Baba *et al.*, an important host factor that needs to be considered is the difference in the strength and maturation of the immune systems of neonates and adult animals.

The immune response potentials of adult and neonate macaques are likely to be different, such that antigen-presenting cell (APC) function for generating strong cellular immune responses could be deficient in the neonates, as they are in healthy human infants younger than 1 year of age (*2*). This type of deficiency could result in an inability or reduced ability of the cellular arm of the immune system to control the extent of replication of the attenuated virus. In contrast, the competent immune system of the adult animals would be expected to hold the attenuated SIV in check, and could result in protection against challenge with wild-type SIV. Such a defect in the APC function of neonates could permit viral replication and the generation of viral products that might be responsible for $CD4^+$ T cell depletion. It has been reported that after priming in the presence of interleukin-12, human naïve neonatal $CD4^+$ T cells appear to develop a T helper cell zero (T_H0) phenotype, whereas adult naïve $CD4^+$ T cells develop into T_H1 cells (*3*). If this difference exists in macaques as well, it could contribute to differences in the immune potential between neonates and adults.

Concerning safety in the use of these attenuated viruses as vaccines, it might be argued that an attenuated HIV vaccine would be safe to use in adults whose immune systems are adequate to control a gene-deleted virus. However, the question remains as to whether the immune system of adults would continue to be sufficiently competent to hold the attenuated virus in check. Other infections, immune-suppressive drug therapy for other conditions, and aging could render the immune system inadequate to control the attenuated virus. Therefore, it is important to determine whether adult macaques that have been infected with attenuated SIV for an extended time will continue to maintain normal CD4 counts and remain without symptoms after immune suppression is induced.

Finally, central nervous system damage poses an additional safety issue for HIV vaccines that may be particularly relevant for infants. HIV-1 is frequently associated with neurologic and behavioral conditions in infants and children, in whom the virus may infect astrocytes as well as microglia, with the *nef* gene implicated in contributing to neuropathologic damage (*4*). Thus, it would be of value to have assessed neuropathologic and neurovirologic parameters at autopsy in the macaques receiving the *nef*, *vpr*, NRE-deleted live SIV vaccine.

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We have reported on the development of a second generation live *nef* attenuated vaccine strain (*1*) based on a gain-of-function approach that could address the safety issues raised by Baba *et al.* This concept is exemplified by the addition of a conditionally lethal suicide gene to a *nef*-deleted (loss-of-function) vaccine strain. Preliminary results suggest that the gain of a conditionally lethal function on top of a loss of a critical virus gene for growth further reduces virus load and perhaps affords greater safety. While we have worked on one prototypic effector gene [herpes simplex virus–1 (HSV-1) thymidine kinase], we realize that many other effectors are possible in attenuating a live vaccine through gain-of-function. Issues concerning a neutral or even a positive selective force needed to maintain a gain-of-function must be addressed before this approach is feasible. Genetic strategies for accomplishing this selection exist, and future refinements on a gain-of-function approach are likely.

Gain-of-function should be considered with loss-of-function in designing safe live attenuated HIV-1 vaccines.

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If SIVΔ3 is pathogenic in macaque infants (*1*, *2*) then, by analogy, HIV with deleted genes might be pathogenic in human infants. Infants must not, therefore, be vaccinated or exposed to these potentially pathogenic HIV mutants. As there are no data thus far that *nef*-deleted SIV is pathogenic in adult macaques, the findings of Baba *et al.* only preclude the vaccination of women who might infect their infants or neonates with HIV having deleted genes. The solution may be to immunize immunocompetent men only. A strategy using an effective attenuated HIV vaccine in men could stop the spread of HIV. This strategy would be effective because men infect men, and men also infect women (*3*). Women do not infect women to any degree (*3*), if at all, and men do not infect infants or children under normal circumstances. Finally, women would not transmit HIV to immunized men. The cycle would be effectively broken. Eventually, all new infections except needle-transmitted HIV between women would be stopped by immunizing men only.

It must still be determined if immunized men would shed enough virus to transmit the vaccine virus to women. Transmission of the vaccine strain between men would not be a problem, as any given two men would be immune in this protocol. Male macaques and SIVΔ3 could be used to test this approach to vaccination.

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Response: We demonstrated that SIVΔ3, a mutant of the SIV deleted in the *nef* and *vpr* genes, induced lethal AIDS in two of four macaque neonates infected orally, but remained attenuated in the adult after intravenous (iv) infection (*1*). Persistently high virus loads were seen in infants, in contrast to adults who seroconverted and were culture-positive only in the first few weeks after inoculation (*1*, *2*). To explain this differential pattern of viremia and pathogenicity, we introduced two new concepts: The threshold hypothesis and a working definition for retrovirus attenuation (*1*, *3*).

According to the threshold hypothesis, retroviral pathogenicity can only become apparent after the virus replicates above a threshold in a given host (Fig. 1). If virus replication is repressed by any mechanism or mechanisms, disease will not ensue, *even if the virus contains the gene or genes that encode virulence*. We postulate that, in macaque neonates, replication of SIVΔ3 was unrestricted and exceeded the threshold, whereas host factors limited replication in adults (*1*, *2*). Although the mechanisms for the differential pathogenicity of SIVΔ3 in adults versus neonates remain to be determined, we agree with Shearer *et al.* that decreased cellular immune responsiveness in neonates may play a major role. Alternatively, activation of the neonatal immune system by encounters with environmental antigens could favor the replication of a *nef*-deleted virus. In vitro studies have shown that Nef augments HIV-1 replication in primary unstimulated T cells, but has no effect in stimulated T cells or permanent T cell lines (*4*).

We propose to classify attenuated viruses into two broad categories according to the *mechanism of attenuation*: Replication-impaired viruses and avirulent viruses, which would not cause disease even in immunocompromised hosts, regardless of their replicative capacity (Table 1). Factors that upregulate virus replication could compensate for the relative loss of replicative power in replication-impaired retroviruses, and virulence could be restored once the threshold is exceeded. Attenuated viruses may also exhibit a mixed pattern within a wide spectrum of partial replication impairment and partial loss of pathogenicity. As SIVΔ3 caused AIDS in macaque neonates, *nef* is not the major molecular determinant for virulence; rather, this gene modulates virus load and influences pathogenicity only indirectly. Other studies demonstrated that the *vpr* gene does not control virulence either (*5*, *6*). Consequently, SIVΔ3 is classified as a replication-impaired rather than avirulent virus.

Other data support the threshold hypothesis. In SCID (severe combined immunodeficiency disease) mice with human fetal thymus transplants, nef^+ HIV-1 replicated and severely depleted thymocytes within 6 weeks, whereas a similar inoculum of *nef*-deleted HIV-1 revealed little cytopathicity (*7*). However, a tenfold higher inoculum of *nef*-deleted virus induced thymocyte loss in a significant fraction of implants by 9 to 12 weeks after infection (*7*).

The threshold hypothesis is confirmed by our studies with Rauscher murine leukemia virus (RLV) (*8*). First, a short course of post-exposure antiviral therapy prevented viremia and disease in all mice inoculated with a high RLV dose, and most resisted rechallenge by the same viral dose without further drug therapy. Thus, pathogenic RLV acted as a pharmacologically attenuated, live virus vaccine (Fig. 1, arrow). Second, normal mice were inoculated with low doses of pathogenic RLV which were still infectious in nude, athymic mice. No antiviral therapy was given. Normal mice without signs of infection were rechallenged with 20 animal infectious doses of RLV; 21% were immune, indicating that protective antiviral immune responses can be generated with a fully pathogenic virus, as long as replication remains below threshold (*9*). Finally, although the pharmacologically attenuated, live RLV vaccine protected many adult mice against high-dose challenge (*8*), the vaccination caused RLV disease when animals were inadvertently co-infected with mouse hepatitis virus (*9*). This experiment reveals a serious danger of vaccine strategies that use replication-impaired live retroviruses: *Success is based on host defenses outracing vaccine virus replication*. Because RLV was only attenuated by drugs interfering with its ability to replicate, the vaccine strategy failed when the animals were temporarily immunocompromised (*9*).

Van Rompay *et al.* discuss SIVmac1A11 (*10*), which has an open *nef* reading frame and several mutations in structural genes which differ from the targeted deletions in SIVΔ3 (*11*). The low virus loads and lack of disease in macaque neonates infected iv with SIVmac1A11 indicate replication impairment and do not contradict our data with *nef*-deleted SIVΔ3, which we classify as replication impaired rather than avirulent. Whether SIVmac1A11 is attenuated also for virulence remains to be determined.

Other issues raised by Van Rompay *et al.* need clarification. They imply that SIVΔ3-infected macaque infants died faster than

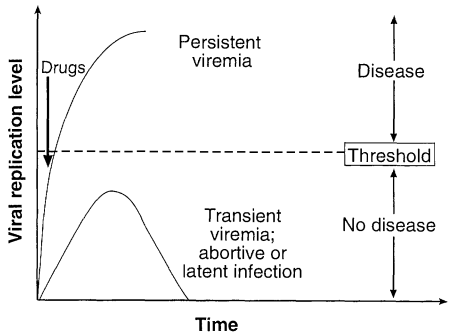

Fig. 1. Host-retrovirus interactions. Development of disease after infection with a retrovirus occurs only after the level of virus replication has exceeded the threshold. Even a fully pathogenic virus can exhibit a pattern of transient viremia and abortive or latent infection if the level of replication does not reach the threshold. Host defenses, antiviral drugs (arrow), or immunotherapy could be used to restrict virus replication soon after infection.

Table 1. Mechanisms of retroviral attenuation. Theoretically, a retrovirus can be attenuated for its ability to replicate, while retaining the gene or genes encoding pathogenicity. Such a virus can become dangerous if the relative loss of replicative power can be compensated by other factors. An avirulent virus, on the other hand, will not cause disease even if the virus is fully replication competent. Consequently, no disease-defining threshold for the level of viral replication exists for this avirulent virus in the host (parentheses).

Mechanism of attenuation	Virus designation	Replication above threshold		Disease	
		In normal host	In co-infected or immuno-compromised host	In normal host	In co-infected or immuno-compromised host
Decreased ability to replicate	Replication-impaired	No	Yes	No	Yes
Loss of pathogenicity	Avirulent	(Yes)	(Yes)	No	No
None	Pathogenic	Yes	Yes	Yes	Yes

their infants infected with parental SIVmac239, which died at 34 and at more than 54 weeks of age (*12*). The time to death within our series was 34 weeks and 46 weeks, respectively (*1*), and two infants are still alive at 19 months of age. Van Rompay *et al.* mistakenly infer that we only saw spuriously low $CD4^+$ T lymphocyte counts in our macque infants. It is important to consider that $CD4^+$ T cell counts are considerably higher in normal macaque infants than in adults. In human infants less than 1 year of age, $CD4^+$ T cell counts of less than 1500 per microliter are abnormal (*13*). Infant 93-7 had less than 500 $CD4^+$ T cells per microliter in every assay (*1*), and animal 94-4 had persistently low $CD29^+CD4^+$ T cell subsets, inverted $CD4^+/CD8^+$ T cell ratios, and 188 $CD4^+$ T cells premortem. Of the two survivors, one has been thrombocytopenic for a year with $CD4^+$ T cell counts that are persistently low for its age, and both have depleted $CD29^+CD4^+$ T cell subsets. Although route of exposure to SIVmac239 did not influence virulence in infant macaques, Van Rompay *et al.* did not address the differential pathogenicity of nef^- mutants in iv infected *adults* and orally infected *infants*. Our preliminary data indicate that age, rather than route of virus exposure, determines SIVΔ3 virulence.

The clinical course and cause of death in our SIVΔ3-infected macaque infants resembled those in macaque infants infected orally with wild-type SIVmac251 (*14*). Typically, these infants do not die of opportunistic infections in our biocontainment facility and do not develop gross neurological dysfunction. The spectrum of disease, that is, immunodeficiency, thrombocytopenia, anemia, and renal disease, is seen also in HIV-1–infected children (*13*). We diagnosed hemolytic anemia in macaque infant 93-7 by persistent reticulocytosis and erythroid hyperplasia in the bone marrow in the absence of overt and occult blood loss, and only confirmatory, by the Coombs test. Large platelets on peripheral blood smears and megakaryocytosis in the bone marrow of the thrombocytopenic infant 93-7 are consistent with peripheral platelet destruction.

We raised the issue of potential adventitious pathogens (*1*); to rule out infection with known simian retroviruses, serial serological testing was performed and diagnostic PCR assays were developed for simian T lymphotropic virus–type I (STLV-I) and simian type D retroviruses (SRV/D). The sensitivity for the latter has been increased; we now detect 0.7 to 1.0 proviral DNA copies of SRV/D serotypes 1, 2, and 3 in 150,000 cells (*15*). Serial samples available from the experimental mother/infant pairs were subjected to a blinded re-analysis; no SRV/D sequences were found. Neither SRV/D isolation, serology, nor PCR analysis are described routinely in the SIV literature. We propose that all macaques be prescreened by SRV/D serology and PCR prior to enrollment into SIV studies.

At first glance, the vaccination strategy suggested by Marx may appear to provide a simple solution; by administering live attenuated HIV-1 to immunocompetent men only, the virus transmission cycle would be broken effectively. We wish to address several potentially serious problems with this strategy.

First, without an animal model for HIV-1 virulence, mutant viruses attenuated for their ability to replicate may have residual virulence that would become manifest only in humans. The relative loss of replicative power of such vaccine viruses could be compensated by various mechanisms, including co-infection with other pathogens, ultraviolet radiation, temporary loss of immunocompetence due to intercurrent illness, or aging. If vaccine virus replication exceeds threshold, AIDS may develop, even in adult men. Safety studies with *nef*-deleted viruses in adult macaques have been limited, and the effects of immunosuppression or immune activation on vaccine virus replication are unknown. Second, the generation of protective responses after vaccination with live attenuated retroviruses depends on adequate levels of replication; if a vaccine virus is too weakened, protection from wild-type pathogenic virus will not be achieved (*16*). Even relatively low levels of viral replication carry the risks of insertional oncogenesis (*17*) and generation of mutant viruses. Disseminated lymphoproliferative disease was reported in an adult macaque 29 months after infection with *nef*-deleted SIV (*18*). Third, all adult rhesus monkeys given *nef*-deleted SIV mutants replicated vaccine virus to high levels for several weeks after vaccination (*1, 2*). During similar initial peaks of viremia after vaccination with *nef*-deleted HIV-1, virus could be transmitted to women who could pass the infection to their children. Ho and Cao (*19*) reported that a woman, identified as HIV-1-infected because she delivered an infected infant, had no disease for more than 12 years. Even though the virus isolated from this mother was attenuated in cultured cells, her child died at age 12 of AIDS. Possibly, the child's virus evolved from an attenuated to a more virulent form, or alternatively, the "attenuated" HIV-1 was more virulent in the susceptible young host, as we demonstrated in macaques (*1*).

Lastly, protective mechanisms following vaccination of adult macaques with SIVΔ3 are slow to develop (*2*); only animals rechallenged with wild-type virus 79 weeks after vaccine administration were protected, but not those re-challenged at 8 or 20 weeks. Slow development of protective responses after vaccination could be dangerous in human vaccine recipients. A false sense of security could lead to increases in risk behavior, thus increasing the chance of wild-type HIV-1 infection during the long time period required for protective responses to mature.

Could *nef*-deleted lentivirus vaccines be made safer? Kestler and Jeang describe a novel concept to increase vaccine safety; a conditionally lethal suicide gene (gain-of-function) will be cloned into the position of *nef*. While currently no in vivo safety and efficacy data are available to evaluate this approach, *nef*-deleted SIV tends to further delete sequences close to the original deletion (*1, 20*); selective pressure may need to be exerted to retain a conditionally lethal suicide gene. Whether a generally applicable vaccine strategy can be derived from this approach, using a virus genome with residual virulence, is not known.

In agreement with Shearer *et al.*, we feel it is premature to consider *nef*-deleted viruses safe, even in adults. Many factors could disturb the fine balance between the rate of vaccine virus replication and the ability of host defenses to contain replication; the tug-of-war between these two opposing influences could be decided in favor of virus replication in the presence of other pathogens or disturbances in the host immune system. Meanwhile, research with *nef*-deleted viruses should proceed to determine the correlates of protection. Even if these viruses are unsafe as human anti-AIDS vaccines, important insights into the mechanisms of resistance to infection with wild-type virus can be gained. After the protective principles are identified, safer vaccine strategies can then be tested for their ability to induce the same host responses.

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Mechanisms of Cardiac Fibrillation

Many mechanisms have been proposed to explain ventricular fibrillation, which is the precursor to sudden cardiac death, the leading cause of death in the industrialized world (*1*). A recent hypothesis discussed by Arthur T. Winfree (*2*) involves three-dimensional (3D) rotors of electrical activity that become unstable when the heart thickness exceeds some critical value. Winfree (*2*, p. 1006) states, "Several pinned rotors would collectively resemble fibrillation in the . . . electrocardiogram, and individual epicardial electrodes would still reveal their individual local periodicities." Although attractive, such an idea remains speculative. Even the most sophisticated systems record extracellular potentials from only a limited number of sites (*3*), making the demonstration of multiple rotors during fibrillation difficult. Moreover, to our knowledge, experimental data are not available as yet showing more than two simultaneous rotors activating the ventricles at various frequencies and resulting in fibrillation. We have used voltage-sensitive probes and high-resolution video imaging to record electrical wave propagation on the surface of the isolated rabbit heart during ventricular fibrillation (*4*). Here we present direct experimental evidence that even a single rapidly moving rotor can give rise to electrocardiographic patterns that resemble fibrillation.

In three episodes of fibrillation, video "movies" of the transmembrane potential signal from the ventricular surface demonstrated a single rapidly moving rotor associated with turbulent electrical activity as recorded by an electrocardiogram (ECG) (Fig. 1A). Specifically, as the rotor (top panel) drifted along complex trajectories on the heart surface (middle), the ECG displayed irregular periodicity and morphology (bottom). To complement our experimental studies of surface data, we conducted computer simulations incorporating more realistic 3D heart geometry (*5*). With the appropriate model parameters we observed a rotor moving rapidly through the heart that was similar to our experimental recordings (Fig. 1). These irregular ECGs and their corresponding narrow-banded frequency spectra (Fig. 2, A and B), for both the experiments and simulations, are consistent with previous data obtained during fibrillation (*6*). Furthermore, the width of these frequency spectra can be related to the frequency of rotation of the rotor, the speed of its motion, and the wave speed through the Doppler phenomenon according to the following relationship:

$$1/(1 + v_{s,max}/v) < f'/f_s < /(1 - v_{s,max}/v) \quad (1)$$

where $v_{s,max}$ is the maximum speed of the rotor, v is the wave speed, and f'/f_s is the observed frequency normalized to the frequency of the rotor. In the experimental episode presented in Fig. 1A, the ratio $v_{s,max}/v$ was 0.39, and for the simulation (Fig. 1B) $v_{s,max}/v$ was 0.37; the dotted lines in Fig. 2 depict the range of frequencies predicted by the use of Eq. 1. Also, the ratio of periods ahead and behind moving rotors, calculated with the use of recordings from the heart surface, also showed excellent agreement with those predicted by the Doppler effect (Fig. 2C). In contrast to Winfree's hypothesis, the activity at individual sites was irregular with narrow-banded spectra similar to those for the ECGs. Our results suggest that it is the speed of the rotor(s), not their number,

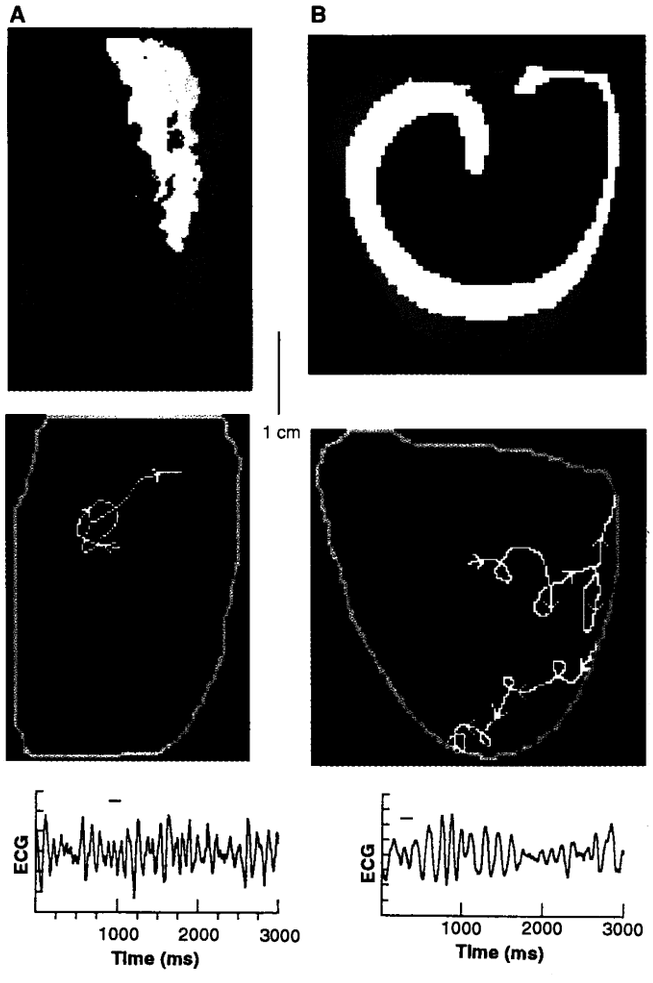

Fig. 1. Rapidly moving rotors. (**A**) Experiment. (**B**) Simulation. Isochrone maps of the surface activity displayed rotors as shown in the top panels. Each isochrone map was computed from one cycle of rotation as denoted by the horizontal bar in the bottom panel (red denotes earliest and purple latest time of activation). Portions of the paths of the organizing centers (cores) of the nonstationary rotors are depicted in the middle panels. Trajectories of the rotor cores were calculated from the surface recordings using time-space plots (*4*). Speed of the rotor cores ranged from zero to approximately 40% of the wave speed. These rapidly moving cores resulted in irregular electrocardiograms that are characteristic of fibrillation as shown in the bottom two graphs.