tibodies neutralize VSV (*18*). It may be that the physicochemical properties of mouse serum and tissues in vivo are drastically different from the buffered saline conditions usually used in vitro. In particular, the kinetics of virus neutralization may be considerably slower in vivo than in vitro, because of complex diffusion kinetics of antibodies in blood and tissue lesions. Because the host-virus interaction is essentially a nonequilibrium system, these complex kinetics and their changes may drastically alter the net outcome of infection.

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5. The mAbs VI22, VI42, VI41, VI46, VI24, VI49, VI30, VI7, VI29, VI40, VI10, and VI32 (*6*) are representative for the steady-state day 12 and day 150 IgG response against VSV-IND. The mAb 25G9 [L. Lefrancois and D. S. Lyles, *Virology* **121**, 157 (1982)] was provided by L. Lefrancois. The IgM mAbs G7G9E4 and G14H3D7 were generated from mice infected with 2×10^6 PFU of VSV-IND 4 days previously.
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12. U. Kalinke *et al.*, *Eur. J. Immunol.* **26**, 2801 (1996). Although recombinant single-chain Fv antibody fragments protect against VSV infection if preincubated in excess with the virus, they are not able to protect in a passive immunization setting, because their half-life is too short.
13. Avidity, neutralizing capacity, and neutralization rate constant as a measure for the on-rate were determined as described (*6*). In brief, avidity was determined from unmanipulated hybridoma supernatants with a solid-phase ELISA, in which intact virus particles were coated. Within a factor of 2, the measured avidities of the antibodies were largely independent of incubation temperatures at 22° and 37°C. The avidities of some antibodies were validated with an in-solution competition assay by determining the concentration of viral glycoprotein in solution required for half-maximal competition of antibody binding in the solid-phase ELISA (*6*). Neutralization rate constants were determined using the kinetics of virus neutralization as read-out. These values reflect physicochemical on-rates. Because VSV is polyvalent, the neutralization rate constant might give slightly higher values than the on-rate [J. Foote and H. Eisen, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1254 (1995)].
14. A VSV-IND variant was generated by growing 10^8 PFU of VSV-IND in the presence of mAb VI22 (150 μg/ml). The obtained virus was plaque-purified in the presence of mAb VI22 and subsequently grown in the presence of mAb VI41 (50 μg/ml). The obtained virus (VSV-TF) was then plaque-purified.
15. M. F. Bachmann, U. Kalinke, A. Althage, G. Freer, C. Burkhart, H.-P. Roost, M. Aguet, H. Hengartner, R. M. Zinkernagel, data not shown.
16. U. Steinhoff *et al.*, *J. Virol.* **69**, 2153 (1995); U. Müller *et al.*, *Science* **264**, 1918 (1994). Infection of $IFN\alpha\beta R^{-/-}$ mice with 10^4 PFU of VSV causes their death by day 2 to 3; protective concentrations of neutralizing antibodies extend survival to day 4 to 7. Because 1 PFU reaching nervous tissues will eventually be lethal, antibody concentrations about 5 to 10 times the protective concentration will usually prevent death after day 7.
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18. Neutralization requires covering of 30 to 50% of neutralizing sites (*11*). This is also illustrated by the observation that neutralization of VSV disappears within a 1:2 to 1:4 dilution step of the antibody, and in vivo protection vanishes in less than a 1:10 dilution step. This covering either blocks docking of VSV to receptors or inhibits fusion or pH-dependent conformational changes in the endosomes. All these mechanisms should operate similarly in vitro and in vivo. In this study, only the IgG2a-subclass antibodies analyzed confirm the conclusions drawn from all neutralizing IgG antibodies. Thus, the results seem not to be attributable to differences in specificity, nor to Fc and complement binding mechanisms, phagocytosis, or aggregations.
19. VSV-IND–specific neutralizing antibody titers were determined as described [M. F. Bachmann *et al.*, *J. Virol.* **67**, 3917 (1993)]. Viral titers in brains were determined as described (*9*). In brief, mice were exsanguinated under ether anesthesia and brains were removed aseptically. Brains were homogenized and viral titers were determined on Vero cells. To exclude the possibility that serum antibodies neutralized VSV particles in the brain after removal of the organ, we mixed VSV into the homogenized brain of a protected mouse and determined the viral titer. No reduction of VSV titer attributable to antibody in the brain was found. BALB/c (SCID) mice were obtained from the Institut für Zuchthygiene (Zürich, Switzerland) or from GSF GmbH (Oberschleissheim, Germany). Animal experiments were performed in accordance with Swiss federal law requiring use of minimal numbers of animals. VSV-IND (Mudd-Summers isolate) was originally obtained from D. Kolakofsky, University of Geneva.

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Bacterial Interference Caused by Autoinducing Peptide Variants

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The synthesis of virulence factors and other extracellular proteins by *Staphylococcus aureus* is globally controlled by the *agr* locus, which encodes a two-component signaling pathway whose activating ligand is an *agr*-encoded autoinducing peptide. The cognate peptides produced by some strains inhibit the expression of *agr* in other strains, and the amino acid sequences of peptide and receptor are markedly different between such strains, suggesting a hypervariability-generating mechanism. Cross-inhibition of gene expression represents a type of bacterial interference that could be correlated with the ability of one strain to exclude others from infection or colonization sites, or both.

"Bacterial interference" refers to the ability of one organism to interfere with the biological functioning of another. Although interference has been assumed to involve growth inhibition, this has been demonstrated in only a few instances (*1*, *2*). We now describe a type of bacterial interference in staphylococci that does not involve growth inhibition, but rather is mediated by inhibition of the synthesis of virulence factors and other extracellular proteins. Expression of the genes encoding these proteins is coordinately controlled by the *agr* locus (*3*–*5*) (Fig. 1), which consists of two divergent transcription units driven by promoters P2 and P3. The P3 transcript RNAIII, rather than any protein, is the effector of the *agr* response, which involves the up-regulation of genes encoding secreted proteins and down-regulation of genes encoding surface proteins (*5*, *6*). The P2 operon contains four genes—*agr*B, D, C, and A—all required for transcriptional activation of the two *agr* promoters (*4*). AgrC corresponds to the signal receptor and AgrA to the response regulator of a standard two-component signal transduction pathway (*4*). AgrB and D generate an autoinducing peptide that is secreted by the bacteria, can be isolated from culture supernatants, and is the activating ligand for AgrC (*7*). Addition of the autoinducing peptide to an early exponential phase culture of the producing strain causes the immediate activation of transcription from the two *agr* promoters (*7*).

The existence of a form of bacterial interference involving this peptide was sug-

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gested by a test of naturally occurring staphylococcal strains for the production of signal molecules activating *agr* transcription in a standard strain, RN6390B. Surprisingly, although all of the strains showed autologous *agr* activation by their own culture supernatants, in many instances these supernatants inhibited rather than activated *agr* expression by RN6390B (8). We then tested a set of seven strains for the effects of their culture supernatants on the same and on different strains, with respect to the *agr* response (Fig. 2, A and B). Because the time course of *agr* activation varied from strain to strain (8), the tests used either early (EEP) or mid (MEP)-exponential phase cells. On the basis of the cross-activation or -inhibition revealed by these tests, *Staphylococcus aureus* strains could be divided into three groups, a group being defined as a set of strains showing mutual cross-activation of the *agr* response (Fig. 2C). In all instances, members of one group inhibited *agr* expression by members of the other two. Non–*S. aureus* strains generally inhibited the *agr* response of *S. aureus* strains from each of the three groups (8).

These results led us to clone and sequence the *agrBDC* regions from three group II and two group III strains plus a second group I strain, using primers flanking *agrBDC* (9). A compilation of the predicted AgrBDC sequences from one strain of each *S. aureus* group plus that from *S. lugdunensis* (10) is shown in Fig. 3. The corresponding sequences for the two or three strains within each group were identical (11), whereas those for strains from different groups were highly divergent between amino acid positions 34 (AgrB) and 205 (AgrC).

To determine whether an *agrD*-encoded peptide was responsible for both the activation and inhibition activities exhibited by culture supernatants, we used a group II and a group III *agrBDC* clone plus the original RN6390B (group I) *agr* clone (7) and a polymerase chain reaction (PCR) product containing *S. lugdunensis agrBD* genes to prepare *agrBD* clones that were introduced into an *agr*-null host strain, RN6911 (5, 9). Supernatants from each of these clones showed the same activity as that of the supernatant from the corresponding parental strain (8), confirming that the *agrBD* complex was responsible for the *agr*-inhibiting as well as the *agr*-activating activity of these strains.

We then purified and sequenced the active material from the supernatants of RN6911 derivatives containing these *agrBD* clones and isolated from each a single active peptide, which showed the same autologous activation and heterologous inhibition as the crude supernatant from which it was purified (Fig. 2D). The four peptides varied in length from seven to nine amino acyl residues and had highly divergent sequences with the exception of a conserved cysteine five residues from the COOH-terminus (Fig. 3B). Sequences of the corresponding *agrD* loci (Fig. 3B) confirmed that each of the active peptides, like that of RN6390B (7), was processed from within the (predicted) AgrD peptide. Synthetic peptides corresponding to the sequences of the RN6390B and *S. lugdunensis* autoinducers, however, had no detectable activity. Mass spectroscopy showed that the synthetic peptides were dimeric, whereas the native peptide molecules were monomeric and had molecular masses that were 18 ± 1 atomic mass units less than those predicted by their respective amino acid sequences (7, 8).

Taken together, these results suggested that the cysteines in the synthetic peptides had spontaneously formed intermolecular disulfides, whereas those in the native peptides were involved in an intramolecular bond, most likely a cyclic

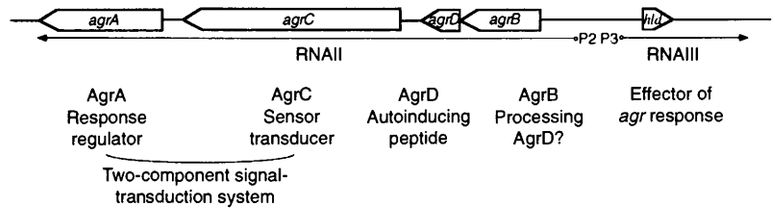

Fig. 1. The *agr* locus of *S. aureus*. Schematic map of the *agr* locus showing the major transcripts RNAII and RNAIII (arrows) and the genes indicated by boxes (3–7).

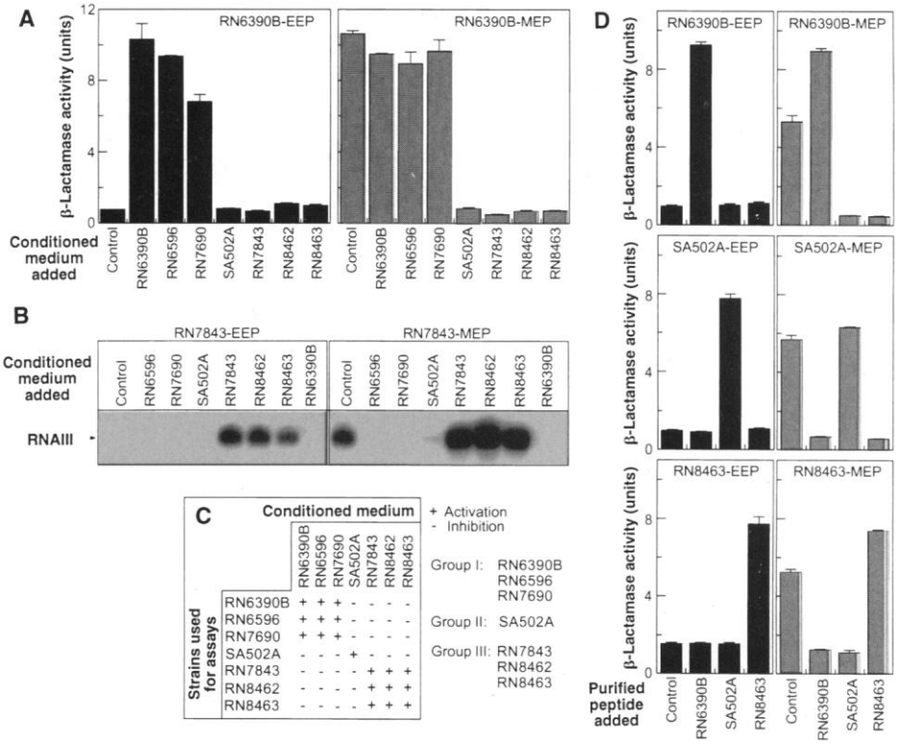

Fig. 2. (A to C) Effects of conditioned media on the transcription of RNAIII in different *S. aureus* strains. Conditioned media from cultures of various *S. aureus* strains (9) were prepared (7). (**A**) RN6390B RNAIII transcription was measured as described (Table 1). (**B**) Conditioned medium (10%) (or CYGP medium as control) was added to each culture of RN7843. After 30-min (for EEP) or 60-min (for MEP) incubation at 37°C, whole cell lysates were prepared and used for Northern blot (RNA) hybridization with a ^{32}P-labeled RN6390B RNAIII-specific DNA probe (7). The blots were exposed to x-ray film (Kodak). For strains RN6596, RN7690, SA502A, RN8462, and RN8463, similar experiments were performed (8). (**C**) Summary of the data in (A) and (B). (**D**) Effects of purified peptides on the RNAIII transcription in different strains. The P3 promoter regions of SA502A and RN8463 were sequenced (12). The DNA sequences of these two promoters are identical to that of RN6390B (4), so the RN6390B *agr* P3-*blaZ* construct was used. The peptides of RN6390B, SA502A, and RN8463 were purified from cells containing the cloned *agrBD* genes (9) as described (19). RNAIII transcription was measured as described (Table 1) with 20 mM tris-HCl (pH 7.5) as control.

thioester introduced posttranslationally and including the COOH-terminal carboxyl, because there is no other conserved carboxyl group in the molecule. Consistent with this possibility were the results of treatment of the native peptides with iodoacetic acid and hydroxylamine. Iodoacetic acid, expected to react with free –SH groups, had no effect, whereas hydroxylamine, expected to react with thioesters, abolished activity (*12*). We recently synthesized a small quantity of a cyclic thioester derivative of the RN8463 (group III) octapeptide and showed that the synthetic material, still impure, inhibits *agr* expression by RN63909B (group I) (*13*). We have not demonstrated *agr* activation with this material and are currently preparing a larger batch to enable better purification.

Table 1. Complementation analysis of *agrB* and *agrD* genes on the production of peptide activity among three groups of *S. aureus* and *S. lugdunensis*. *Staphylococcus aureus* RN6390B (pRN6683, containing RN6390B *agr* P3-*blaZ* fusion) (*4*) cells were grown in CYGP medium at 37°C to EEP or MEP. To each culture, 10% conditioned medium prepared (*7*) from *S. aureus* cells containing cloned *agrB*, or *agrD* or various combinations from representative strains of three groups of *S. aureus* (I, RN6390B; II, SA502A; and III, RN8463) and *S. lugdunensis* (*9*), or CYGP medium as control, was added. The cultures were incubated at 37°C with shaking. After 55-min (for EEP) or 80-min (for MEP) incubation, β-lactamase activity was measured as described (*7*). +, activation; +*, inhibition; −, no effect.

agrD \ *agrB*	Group I	Group II	Group III	*S. lugdunesis*
Group I	+	−	+	−
Group II	−	+*	−	−
Group III	+*	−	+*	−
S. lugdunensis	+	−	−	+*

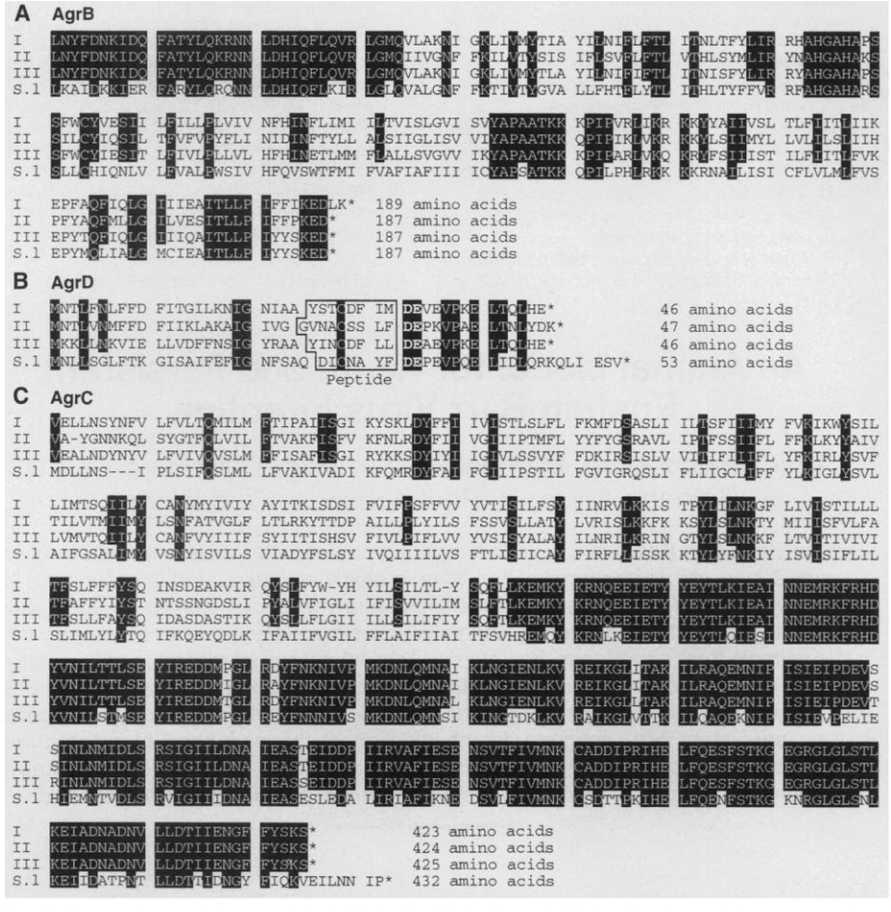

Fig. 3. Comparisons of the predicted (**A**) AgrB, (**B**) AgrD, and (**C**) AgrC amino acid sequences from different *S. aureus* groups and *S. lugdunensis*. The sequences were aligned by MegAlign software (GeneStar). The sequences are as follows: *S. aureus* group I (RN6390B and RN7690) (*4*), II (SA502A, RN6923, and RN6925) (GeneBank accession number AF001782), and III (RN8462 and RN8463) (GeneBank accession number AF001783); and S.I (*S. lugdunensis*) (*10*). Within the same group, the AgrBDC sequences are identical. Residues identical in all four or in all three groups of *S. aureus* are shaded. Dashes indicate gaps generated by the align program. In (B), the sequences of the peptides are boxed and the conserved aspartate-glutamate are in bold. Asterisks indicate stop codon. Single-letter 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.

Similarly, AgrC has diverged in parallel with AgrD so as to retain the specificity of the receptor-ligand interaction that leads to activation. Here, the divergent region is the NH_2-terminal half, which was predicted by sequence analysis and confirmed by PhoA fusions (*15*) to span the cytoplasmic membrane and which must contain the site of interaction with the different peptides. The COOH-terminal half, which contains the conserved histidine and transmits the autophosphorylation signal (*16*), is highly conserved.

The sequence divergence is confined to the region of the *agr* locus that is responsible for the specificity of processing and of the ligand-receptor interactions, and the junctions between the conserved and divergent regions are very sharp, at the nucleotide as well as at the amino acid level. This

The cyclic thioester bond is probably introduced during processing, and we suspect that AgrB is responsible for this step and possibly for secretion as well. The predicted AgrB sequences (Fig. 3A) suggest that AgrB is a transmembrane protein, and we have confirmed this by means of PhoA fusions (*12*). Except for the first 34 amino acyl residues, AgrB is divergent, and we have shown by analysis of separate *agrB* and *agrD* subclones that AgrB determines the specificity of AgrD processing. An *agr*-null strain containing the cloned group I *agrB* and group III *agrD* produced group III autoinducer activity, and vice versa (Table 1); no other heterologous combinations were active, although each of the parental *agrBD* pairs generated the expected activity. We conclude that despite the sequence divergence of *agrB* and *D*, the two gene products have retained the specific interactions that are required for maturation of the peptide derivatives of AgrD. Genetic and in vitro biochemical analyses to determine the precise role of AgrB and the different roles of the conserved and divergent regions of the protein are in progress. A single transmembrane protein is responsible for processing and secretion of peptidic bacteriocins by lactococci (*14*); however, this protein is unrelated to AgrB and is much larger.

type of sequence organization is similar to that of the exchangeable cassettes that are responsible for antigenic switching and other types of phase variation in microorganisms (*17*). It would not be surprising if some sort of cassette-switching mechanism were responsible for exchanging these divergent gene segments between strains or species, or both. The evolutionary mechanism for divergence in concert, however, remains unknown and may involve a hypervariability-generating system.

Studies are presently directed toward the biological role or roles of these groupings. Group-specific differences in the expression of subsets of virulence factors or other extracellular proteins could be related to differences in disease patterns. Group-specific differences in the expression of colonization factors could be related to interstrain interference with colonization (*1*) and to differences in colonization site preference. Indeed, we have observed that the vast majority of menstrual toxic shock strains belong to *agr* group III and are characterized by a coherent overall biotype (*12*). This finding could reflect a tissue tropism for the human vaginal mucosa; however, it is inconsistent with the fact that many surface proteins, presumably including colonization factors, are down-regulated by *agr* (*18*). Finally, ligand-based inhibition of virulence or colonization factor expression, or both, could represent the basis of a therapeutic or prophylactic initiative that may not be limited to staphylococci.

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9. *Staphylococcus aureus* strains used: RN6390B (*5*), RN6596 [American Type Culture Collection (ATCC) 27733], RN7690 (V8), SA502A (ATCC27217), RN6923, and RN6925 (methicillin-resistant clinical isolates); and RN7843, RN8462, and RN8463 (clinical toxic shock syndrome isolates). PCR products were prepared in which chromosomal DNAs from these strains were used as templates and oligonucleotides flanking the *agrBDC* of RN6390B (*4*) were used as primers. For strains RN7690, RN6923, RN6925, and RN8462, the PCR products were directly sequenced with an automated DNA sequencer (Molecular Dynamics), and sequencing was continued with the primer walking strategy. For SA502A and RN8463, the PCR fragments were cloned into plasmid pRN5548 (*5*) and sequenced. *Staphylococcus lugdunensis* RN8160 (*10*) *agrBD* strains were constructed by cloning a PCR product with oligonucleotides flanking the *agrBD* genes as primers and chromosomal DNA as template into pRN5548. pRN5548-RN6390B *agrBD* was constructed as described (*7*). The *agrBD* genes of SA502A and RN8463 were cloned by deleting *agrC* from pRN5548-*agrBDC* constructs. Plasmids used in Table 1: pRN5548-RN6390B *agrD* (*7*) and pRN6441-RN6390B *agrB* were constructed by cloning a Cla I fragment of plasmid pRN6912 (*7*) into pRN6441 (*4*); for other strains: pRN5548-*agrD* plasmids were constructed by in-frame internal deletion of *agrB* genes from pRN5548-*agrBD* plasmids; pRN6441-*agrB* plasmids were constructed by first deleting the *agrD* genes from pRN5548-*agrBD* plasmids, the resulting plasmids were then digested with Cla I, and the DNA fragments containing *agrD* were cloned into pRN6441. All plasmids were transformed into *S. aureus* RN7667 [RN6911(pI524) (*7*)].
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19. The autoinducing peptide from RN6390B was purified as described (*7*). For others, the peptides were purified from the culture supernatants of RN7667 cells containing cloned *agrBD* genes of SA502A, or RN8463, or *S. lugdunensis* grown in dialyzed tryptophan assay medium (*7*) plus L-tryptophan (50 μg/ml) and 2-(2-carboxyphenyl)benzoyl-6-aminopenicillanic acid (5 μg/ml) starting at 2×10^9 cells/ml. After 6-hour growth, cells were removed by centrifugation and the supernatant was filtered (0.22-μm filter), boiled for 10 min, lyophilized, and suspended in solution A (2.5% acetonitrile plus 0.1% trifluoroacetic acid). This material was loaded onto a Sephasil C18 (Pharmacia) column (3 cm × 5 cm), washed once with solution A, once with solution B (15% acetonitrile plus 0.1% trifluoroacetic acid), and eluted with solution C (40% acetonitrile plus 0.1% trifluoroacetic acid). The eluted material was lyophilized, suspended in 20 mM tris-HCl buffer (pH 7.5), and filtered through a Centricon 3 filter (Amicon). The filtrate was then loaded onto a high-performance liquid chromatography C-18 column and eluted with an acetonitrile gradient (16 to 32%) at 0.2% acetonitrile per minute. Fractions with activity were pooled, lyophilized, suspended in 20 mM tris-HCl buffer (pH 7.5), and analyzed by matrix-assisted laser desorption/ionization–mass spectrometry [F. Hillenkamp, M. Karas, R. C. Beavis, B. T. Chait, *Anal. Chem.* **63**, 1193A (1991) (*7*)], and the amino acid sequence was determined by a Perkin-Elmer Procise Edman Sequencer.

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An Animal Model for Acute and Persistent Epstein-Barr Virus Infection

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Epstein-Barr virus (EBV) is a human lymphocryptovirus that causes infectious mononucleosis, persists asymptomatically for life in nearly all adults, and is associated with the development of B cell lymphomas and nasopharyngeal carcinomas. A highly similar rhesus lymphocryptovirus naturally endemic in rhesus monkeys was used to orally infect naïve animals from a pathogen-free colony. This animal model reproduced key aspects of human EBV infection, including oral transmission, atypical lymphocytosis, lymphadenopathy, activation of $CD23^+$ peripheral blood B cells, sustained serologic responses to lytic and latent EBV antigens, latent infection in the peripheral blood, and virus persistence in oropharyngeal secretions. This system may be useful for studying the pathogenesis, prevention, and treatment of EBV infection and associated oncogenesis.

Despite progress in basic and clinical Epstein-Barr virus (EBV) research, the absence of a suitable animal model for EBV infection continues to impede studies of viral pathogenesis, vaccine development, and therapeutics. EBV infection is typically initiated by transmission in oral secretions, virus replication in the oropharynx, and infection of peripheral blood B lymphocytes (*1*). Primary EBV infection is the most common cause of infectious mononucleosis (*2*). Thereafter, EBV persists as a latent infection in a small fraction of B lymphocytes and replicates sporadically at low levels in the oropharynx (*1*). Virus reactivation and shedding in oral secretions enables completion of the virus life cycle by infection of naïve hosts. Latent EBV infection is generally asymptomatic but can lead to polyclonal EBV-infected B cell lymphoproliferations and lymphomas when individuals are immunosuppressed