pattern on its thick outer (perhaps exine) wall, and its nonseptate germination tube (*12*). Another possible pollen grain or meiospore (25 μm in diameter) that contains two protoplasts is also shown (Fig. 1J).

A possible fungal spore (29 μm in diameter) is represented by the four septate, hyaline, ellipsoid structures in Fig. 1K. Similar spores are found in extant representatives of the genera *Dactylium* and *Hyaloflorae* in the Moniliales. Both of these genera contain saprophytic species. A possible fungal or algal vesicle (37 μm in diameter) with zoospores or an oogonium with oospheres (as in the extant genus *Saprolegnia*) is shown in Fig. 1L.

All of these fossils represent a biocenosis comprising a community of organisms that lived on the resin-bearing plant. Although the host plant could not be identified from analyses of the amber (*13*), it may have been the cycadeoid *Pterophyllum jaegeri* because plant megafossils in the surrounding Raibler Sandstone were identified as belonging to this species. Some of the organisms shown here probably lived on the surface of the bark or leaves of the resin-producing plant (as do extant representatives of *Scytonema* and *Trentepohlia*). During prolonged periods of rainfall, stagnant water would have formed in bark crevices or branch bases long enough for populations of aquatic or semiaquatic microorganisms (such as ciliates, amoebas, and sheathed bacteria) to become established. Ciliates, especially larger forms, are indicators of eutrophic, often mesosaprobic environments (*14, 15*); we may presume a similar, nutrient-rich habitat for the fossil organisms. We speculate that these microhabitats were suddenly inundated with resin from the associated plant. The pollen grains and fungal spores could have fallen into the water source or have been blown against the sticky resin.

Bacteria, fungi, and algae are well known from marine rocks (*16–20*). Isolated bacterial cells have been observed in Tertiary amber (*3, 21, 22*), a ciliate resembling *Paramecium* was reported in Cretaceous amber (*3, 23*), and the test of the amoeba, *Prantlitina*, was reported from freshwater sediments of the Namurian (Carboniferous) of Czechoslovakia (*24*). Molecular studies have suggested that morphological evolution is slow or stationary in several protist groups; morphological stasis has been suggested in *Tetrahymena* (*25*) and in the amoebas *Acanthamoeba* and *Naegleria* (*26*) on the basis of deep protein or nucleic acid sequence differences among essentially identical species or strains. That the fossil ciliates and most of the other microorganisms reported here can be referred to modern groups confirms this morphological stasis as far back as 230 million years.

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Presentation of a Viral T Cell Epitope Expressed in the CDR3 Region of a Self Immunoglobulin Molecule

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Synthetic peptides corresponding to microbial epitopes stimulate T cell immunity but their immunogenicity is poor and their half-lives are short. A viral epitope inserted into the complementarity-determining region 3 (CDR3) loop of the heavy chain of a self immunoglobulin (Ig) molecule was generated from the Ig context and was presented by I-E^d class II molecules to virus-specific, $CD4^+$ T cells. Chimeric Ig-peptide was presented 100 to 1000 times more efficiently than free synthetic peptide and was able to prime virus-specific T cells in vivo. These features suggest that antigenized Ig can provide an improved and safe vaccine for the presentation of microbial and other peptides.

Synthetic peptides can act as antigens for stimulating humoral and cell-mediated immunity. Some problems with the use of peptides as vaccines are short half-lives, poor immunogenicity, and a requirement for Freund's adjuvant (*1, 2*). The antigen-binding or CDRs of Ig molecules represent an array of peptides that are as diverse as T cell epitopes and are also comparable in size. We explored the capacity of self Ig to present known microbial epitopes engineered into a CDR loop, given the additional evidence that self Ig molecules have long half-lives and might also be more efficiently internalized by Fc receptors for Ig on antigen-presenting cells (APCs).

We used the 5.5-kb DNA fragment encoding the heavy chain variable region (V_H) of the 91A3 antibody to arsonate (*3*) in a polymerase chain reaction mutagenesis

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(4) to replace the D segment with a nucleotide sequence encoding a helper T cell epitope of the hemagglutinin (HA) of PR8 influenza A virus as described (4). This epitope corresponds to amino acid residues 110 to 120 of HA and is recognized by $CD4^+$ T cells in association with $I\text{-}E^d$ class II molecules of major histocompatibility complex (MHC) (5). The mutated V_H gene, from which the D segment was deleted and the cognate peptide sequence inserted in the correct frame (6), was subcloned in a pSV2gpt vector (7) upstream of the exons of the BALB/c γ2b constant region from which the MOPC 141 VDJ fragment (7) had been excised. To express this gene with the homologous light chain gene, we transfected the vector into the non–Ig-secreting BALB/c myeloma B cell line SP2/0, together with a pSV2-neo vector (8) carrying the rearranged 91A3 light chain gene (9). The techniques used in the construction of these vectors, the subsequent transfections, and the selection of antibody-secreting transfectoma cells were similar to those we used to generate a chimeric antibody (Ig-NP) that carried an influenza virus nucleoprotein (NP) epitope that was recognized by $CD8^+$ T cells (4). We purified the antibodies produced by cells transfected with chimeric heavy and wild-type light chain genes (Ig-HA) as described (4) and tested them for the ability to stimulate the LD1-24 T cell hybridoma (5) specific for this epitope (site 1) of influenza virus HA (Table 1). Like the synthetic HA(110–120) peptide and PR8 virus, the chimeric Ig-HA induced the activation of LD1-24 T cells. In contrast, the T cells did not respond to the NP(147–161) peptide, which represents an epitope recognized by $CD8^+$ T cells in association with K^d class I antigen (10), or to wild-type Ig, or to chimeric Ig-NP.

Further experiments showed that the presentation of Ig-HA was mediated mainly by means of the Fc γ receptor (FcγR) along a standard endocytic pathway. These conclusions are based on the following findings. First, monoclonal antibody (MAb) to FcγR, 2.4G2 (11), inhibited the presentation of Ig-HA but not the presentation of either PR8 influenza virus or HA(110–120) peptide (Fig. 1). An IgM MAb and an Fab fragment of an IgG1 MAb, both of which do not bind to FcγR, did not inhibit the presentation of Ig-HA. A rat MAb of the same isotype as 2.4G2, and Ig (wild-type 91A3 antibody) that is of the same isotype (IgG2b) as Ig-HA, showed much weaker inhibition, requiring 200 times more protein than 2.4G2. Thus, the strong inhibi-

Table 1. Activation of LD1-24 T helper cells by chimeric Ig-HA. Purified antigens were incubated with 4×10^5 irradiated (2200 rads) BALB/c spleen cells as APCs and 2×10^4 LD1-24 T cells in a total volume of 200 μl in round-bottom 96-well plates for 48 hours as described (5). We assessed T cell activation by measuring interleukin-3 (IL-3) production in the supernatant (50 μl) by means of the colorimetric MTT assay (26) with 15×10^3 cells of the IL-3–dependent DA-1 line as described (5).

Antigen*	Concentration in μg/ml (nM)†	A_{570}‡
None		0.121 ± 0.018
HA(110–120)	71 (50 × 10^3)	0.846 ± 0.017
NP(147–161)	125 (68 × 10^3)	0.148 ± 0.015
Ig-HA	15 (1 × 10^2)	0.852 ± 0.011
Ig-NP	15 (1 × 10^2)	0.148 ± 0.020
Ig	15 (1 × 10^2)	0.150 ± 0.020
PR8	2	0.919 ± 0.060

*The following antigens were used: HA(110–120) peptide, synthetic peptide corresponding to amino acid residues 110 to 120 [SFERFEIFPKI (27)] of HA; NP(147–161), synthetic peptide corresponding to amino acid residues 147 to 161 [TYQRTRALVRTG-MDP (27)] of influenza virus NP; Ig-HA, 91A3 antibody bearing the HA(110–120) epitope in place of the heavy chain D segment; Ig-NP, 91A3 antibody bearing the NP(147–161) cytotoxic T lymphocyte epitope in place of the heavy chain D segment (4); Ig, wild-type 91A3 antibody (4); and PR8 virus (UV)–inactivated influenza A PR8 virus. †The indicated virus concentrations are total viral protein. The molarity of the antigens was calculated with the following molecular masses: Ig-HA, Ig-NP, and Ig with 150,000 daltons; HA(110–120) peptide with 1,428.5 daltons; and NP(147–161) peptide with 1,828.2 daltons. For PR8, the molarity cannot be calculated on the basis of viral protein concentration. The actual content of PR8 virus in HA(110–120) peptide (2 μg/ml) is ~ 7 nM, on the basis of the approximations that 25% of the viral proteins are HA and that each HA molecule is a trimer. ‡Mean ± SD of quadruplicates. The absorbance was measured at 570 nm (reference wavelength of 630 nm). Controls were LD1-24 plus Ig-HA without spleen cells (0.069 ± 0.01) and spleen cells plus Ig-HA without T cells (0.05 ± 0.01).

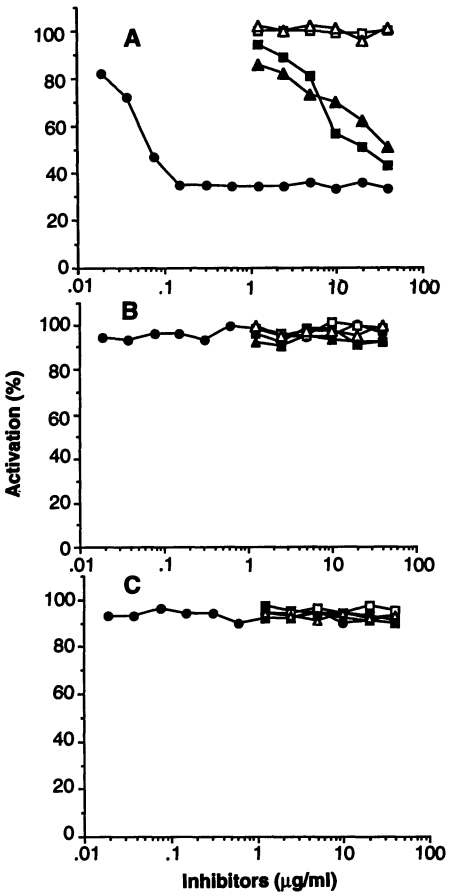

Fig. 1. Inhibition of Ig-HA–induced T cell activation by MAb to FcγR. In these experiments, T cells were stimulated with Ig-HA (**A**) at 2 μg/ml, ultraviolet (UV)-inactivated PR8 virus (**B**) at 1 μg/ml, or 50 μM HA(110–120) synthetic peptide (**C**) as described in Table 1, except that spleen cells were incubated for 1 hour with blocking MAbs before the addition of the antigens. The MAbs used were as follows: 2.4G2 (●), a rat IgG2b MAb to FcγR; rat Ig (■), a rat IgG2b MAb to DNP (dinitrophenyl); Ig (▲), wild-type IgG2b 91A3 antibody produced by SP2/0 transfectoma cells (4); IgM (△), mouse MAb to Sm (Smith antigen); and Fab (□), Fab fragment of an IgG1 MAb. The percent activation was calculated with the following formula: (A_{570} in presence of MAb − background)/(A_{570} in absence of MAb − background) × 100. The A_{570} values in the absence of MAbs were as follows: 0.935 ± 0.02 for Ig-HA (mean ± SD; n = 4), 0.960 ± 0.009 for synthetic peptide, and 0.946 ± 0.03 for PR8 virus, and the A_{570} values in the absence of both antigen and MAb (background) were 0.144 ± 0.014, 0.066 ± 0.007, and 0.09 ± 0.03, respectively.

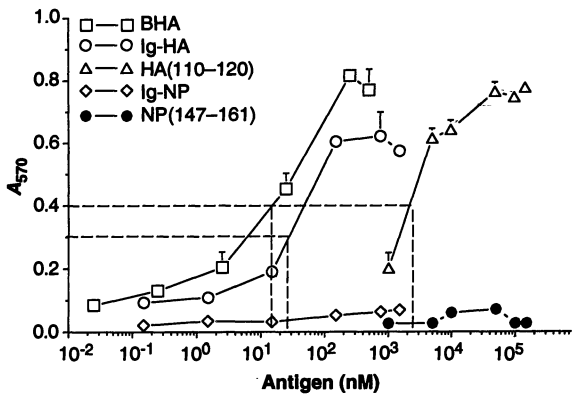

Fig. 2. Efficacy of T cell activation by Ig-HA. These experiments were carried out as described in Table 1 with the use of 1×10^4 dendritic cells as APCs and graded quantities of the indicated antigens. The dendritic cells were purified from the spleen of BALB/c mice (19), and BHA was purified as described (28). The molarity of BHA was calculated with a molecular mass of 200 kD. Each point represents the mean ± SD of quadruplicates after deduction of the mean background (A_{570}, 0.08) obtained by the incubation of the T cells and the APCs without antigen. The dashed lines indicate the amounts of antigens that lead to 50% activation.

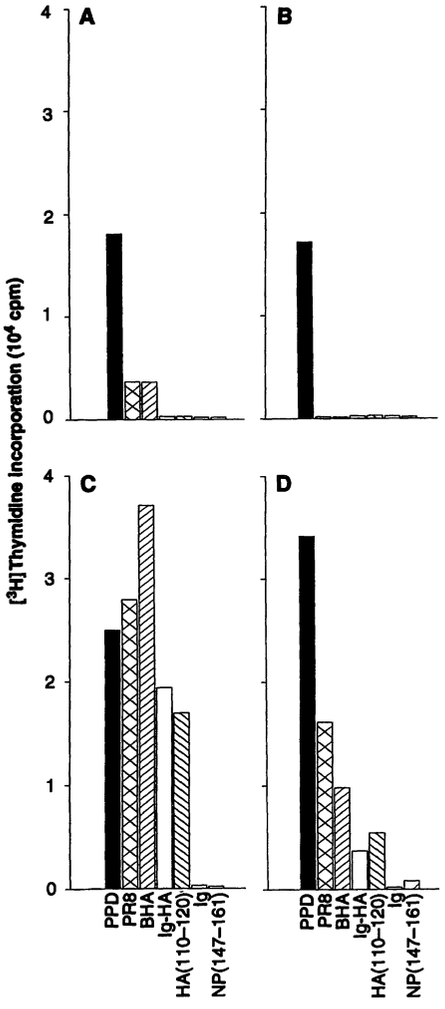

Fig. 3. Priming of specific T cells by Ig-HA in vivo. The proliferative response of lymph node cells from mice immunized with Ig (**A**), NP(147–161) (**B**), Ig-HA (**C**), or HA(110–120) (**D**) were assessed by [^{3}H]thymidine incorporation. BALB/c mice were immunized subcutaneously at the base of the tail with Ig (150 μg), NP(147–161) peptide (100 μg), Ig-HA (150 μg), or HA(110–120) peptide (100 μg) emulsified in 300 μl of phosphate-buffered saline and complete Freund's adjuvant (1:1 v/v). Seven days later, the draining lymph nodes were removed, and the cells were separately cultured in flat-bottom, 96-well, microtiter plates (2×10^5 cells per well) with Ig-HA (1 μg/ml), Ig (1 μg/ml), HA(110–120) peptide (7 μg/ml), NP(147–161) peptide (9 μg/ml), BHA (10 μg/ml), UV-inactivated PR8 virus (10 μg/ml), purified protein derivative (PPD; 10 μg/ml), or no antigen. Cultures were set up in triplicates from pooled lymph node cells of two to three mice, incubated for 5 days under standard conditions, and pulsed for the last 18 hours with 1 μCi of [^{3}H]thymidine per well. Data represent the mean of counts per minute obtained in the presence of antigen minus that obtained in the absence of antigen. In the absence of stimulators, the counts per minute incorporated were as follows: 8767 ± 2420, 8560 ± 2326, 4611 ± 523, and 7297 ± 2505 for the cells of mice immunized with Ig-HA, Ig, HA(110–120) peptide, and NP(147–161) peptide, respectively.

tory effect of 2.4G2 appears to be a result of its high affinity for FcγR (*12*) determined by the 2.4G2 V region encoded binding site rather than by the 2.4G2 Fc region. The lack of total inhibition of T cell stimulation by 2.4G2 may be a result of non-FcγR–mediated internalization of Ig-HA. Second, the presentation of Ig-HA, but not of HA(110–120) peptide, was blocked by chloroquine and by fixation of APCs with paraformaldehyde before peptide presentation (*13*). This indicates that Ig-HA needs to be processed in acidic vacuoles for subsequent recognition by T cells. Third, presentation of both Ig-HA and HA(110–120) peptide was blocked by antibody to I-E^d (anti–I-E^d) but not by antibody to I-A^d (anti–I-A^d), which indicates that the peptide generated from the chimeric Ig-HA is recognized in association with the I-E^d MHC class II molecules, just like the corresponding synthetic HA peptide (*14*).

Because the HA peptide can be generated from Ig-HA protein, which has flanking regions different from those found in the viral HA, we compared the efficacy with which Ig-HA, HA(110–120) peptide, and HA protein were presented. As can be seen in Fig. 2, when dendritic cells, which are potent APCs for T cell–mediated immunity (*15, 16*), were used for presentation, the 50% activation of T cells [absorbance at 570 nm (A_{570}) was 0.3 for Ig-HA and 0.4 for HA purified from bromelain-treated PR8 virus (BHA) and HA(110–120) peptide] required 25 nM Ig-HA, 15 nM BHA, and 2.5×10^3 nM HA(110–120) peptide. Similarly, when spleen cells were used as APCs (*17*), the amounts of Ig-HA, BHA, and HA(110–120) peptide required for 50% activation were approximately 20, 6, and 20,000 nM, respectively.

These results suggest that Ig-HA is presented 100 to 1000 times better than HA(110–120) peptide (25 nM versus 2.5×10^3 nM in the case of dendritic cells and 20 nM versus 2×10^4 nM in the case of spleen cells). Because the equivalent of 1 nM HA(110–120) peptide can be found in 0.5 nM Ig-HA, the amount of HA(110–120) peptide that can be released from 20 and 25 nM Ig-HA would be 40 and 50 nM, respectively. The amounts of generated peptide (probably overestimated, given the imperfect efficiency of protein internalization and processing) that are required for 50% activation are similar to the amounts contained in BHA (18 nM in the case of spleen and 45 nM in the case of dendritic cells, estimated on the basis that 1 nM BHA contains 3 nM peptide) but are at least 1/50 to 1/500 of those required if synthetic peptide is used (2×10^4 nM in the case of spleen and 2.5×10^3 nM in the case of dendritic cells) (Fig. 2). The lower efficacy of HA(110–120) in stimulating T cells cannot be attributed to molecular heterogeneity of the peptide preparation (some species would have higher affinity for I-E^d) because high-performance liquid chromatography analysis of the peptide showed a single peak, corresponding to a 99% degree of purity (*17*).

Therefore, it appears that peptides generated from Ig-HA and BHA in the endocytic compartment subsequent to internalization are much more efficient than synthetic peptide in stimulating T cells. Efficacy may also be due to the generation of a peptide that has a higher affinity or more accessibility to I-E^d molecules, especially newly synthesized class II MHC molecules (*18*). Ig-HA is as efficient as HA, the native, viral protein. Although the two proteins are likely to be taken up by distinct adsorptive receptors (FcγR for Ig-HA and sialic acid–bearing receptors for BHA), it is possible that both Ig-HA and BHA are internalized and processed with the same efficiency and generate identical peptides. On the other hand, dendritic cells required less synthetic peptide than spleen cells but more Ig-HA and BHA. The more efficient presentation of synthetic peptide by dendritic cells may be related to the high density of class II molecules at the surface of these cells as compared with spleen cells (*18, 19*), whereas the requirement of larger amounts of both Ig-HA and BHA (Fig. 2) may be related to poorer internalization or decreased processing of these antigens by the subset of dendritic cells that is purified by the standard plastic adherence protocol (*20*). Furthermore, the density of FcγR on dendritic cells is low (*20, 21*).

Finally, Ig-HA is able to prime a virus-specific T cell response in vivo. This conclusion is supported by the data depicted in Fig. 3, which show that lymphocytes from mice immunized with Ig-HA or HA(110–120) peptide, but not with wild-type Ig or NP(147–161) peptide, proliferate after in vitro stimulation with HA(110–120), Ig-HA, BHA, and PR8 virus. No proliferative response was seen when Ig and NP(147–161) peptide were used as stimulators. The proliferative response was higher in the case of immunization with Ig-HA than with HA(110–120) peptide.

In summary, our data reveal that similar or perhaps identical peptides that bind to class II MHC molecules are generated from a self Ig molecule and a foreign viral protein independent of the flanking regions. This observation is in agreement with data that demonstrate that peptides known to associate with class I MHC molecules and recognized by CD8$^+$ T cells can be generated from flanking regions distinct from their

natural environment (*4, 22–24*) and that peptides for $CD4^+$ T cells can be inserted as guests in protein carriers (*25*).

Because Igs are self components devoid of the side effects sometimes associated with viral vaccines or viral proteins, exhibit a longer half-life than synthetic peptides, and can be internalized into APCs by means of FcγR, self Ig molecules that carry foreign T helper epitopes alone or in combination with a foreign B cell epitope could represent a new type of safe vaccine aimed at stimulating strong, specific immunity. This approach could also be extended to tumor-associated antigens and could represent a strategy to develop reagents able to stimulate antitumor immunity. The potential of chimeric self Ig-peptides as immunogens is enhanced by the fact that these constructs can be presented by dendritic cells, which are APCs known to be capable of capturing antigens in an immunogenic form in situ and of sensitizing naïve T cells in the absence of foreign adjuvants (*15, 16*). Because a T cell epitope can be generated from the CDR3 segment of a self Ig, it is possible that self Ig molecules with CDR3 segments having sequences recognized by T cells can contribute to autoimmune phenomena.

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14. Irradiated (2200 rads) spleen APCs (4×10^5) were incubated with graded quantities of either anti–I-E^d 14-4-4S or anti–I-A^d 25-9-17S. One hour later, Ig-HA (2 μg/ml) and HA(110–120) (5 μM) were added. After 2 hours, LD1-24 T cells (2×10^4) were added, and the mixture was incubated for 2 days. T cell activation was then measured as described in Table 1. In the presence of anti–I-E^d, 84% and 96% inhibition, respectively, of Ig-HA– and HA(110–120)–mediated T cell activation was obtained. No significant inhibition was obtained in the presence of anti–I-A^d.
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Evidence for a Clonal Origin of Methicillin Resistance in *Staphylococcus aureus*

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Soon after methicillin was introduced into clinical practice in the early 1960s, resistant strains of *Staphylococcus aureus* (MRSA) appeared, bearing a newly acquired resistance gene, *mecA*, that encodes a penicillin binding protein, PBP2a. MRSA have spread throughout the world, and an investigation of the clonality of 472 isolates by DNA hybridization was performed. All 472 isolates could be divided into six temporally ordered *mecA* hybridization patterns, and three of these were subdivided by the chromomosomal transposon Tn*554*. Each Tn*554* pattern occurred in association with one and only one *mecA* pattern, suggesting that *mecA* divergence preceded the acquisition of Tn*554* in all cases and therefore that *mecA* may have been acquired just once by *S. aureus*.

Staphylococcal resistance to methicillin (Mc^r), a frequent occurrence in both community and nosocomial settings (*1*), is a prime example of the development and spread of bacterial resistance after the introduction of an antibiotic into clinical practice. In Mc^r in *S. aureus*, a resistance determinant encodes a penicillin binding protein, PBP-2a (*2–4*), that binds β-lactams with much lower affinity than the native PBPs. Because PBP-2a is an addition to the standard set of PBPs, rather than a mutational modification of one of them, and was almost certainly acquired from some other organism after the introduction of methicillin (*5–7*), it presents an opportunity to study the evolution and spread of a single bacterial gene during a clearly defined time frame.

We report here the use of genomic DNA fingerprinting with variable gene (*8*) probes to construct an unambiguous temporally determinate (rooted) evolutionary tree encompassing over 450 Mc^r strains isolated during the past 30 years worldwide. The tree is based on molecular parsimony coupled with the temporal sequence of strain isolation and is consistent with the diversification of methicillin-resistant *S. aureus*

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