subcutaneously with 500 μg of acetone-precipitated baculovirus-expressed adipsin in complete Freund's adjuvant. Animals then received two booster injections of 250 μg of baculovirus-expressed adipsin in incomplete Freund's adjuvant at 6-week intervals. Animals were bled 10 days after the last injection. The antisera so obtained reacted on immunoblots with purified adipsin expressed from baculovirus or mammalian expression systems and with purified human complement D. Immunoglobulin G (IgG) purified from these sera completely inhibited the factor D activity in mouse sera, as assayed by the cleavage of ^{125}I-labeled human factor B, while IgG from nonimmune animals had no effect.
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33. Conditioned medium from stably transfected CHO cells was collected for 48 hours in serum-free Dulbecco's modified essential medium containing .05 m*M* zinc chloride and 1 m*M* dexamethasone. Proteins that precipitated from 60% to saturation with ammonium sulfate were dialyzed against 100 m*M* NaCl, 50 m*M* tris, *p*H 7.5, and applied to a column of concanavalin A–Sepharose 4B equilibrated with the same buffer. Proteins were eluted with 0.5*M* α-methyl-mannoside in 100 m*M* NaCl, 50 m*M* tris, *p*H 7.5, dialyzed against 20 m*M* NaCl, 50 m*M* tris, *p*H 7.5, and applied to a Waters Protein-pak DEAE HPLC column at a flow rate of 1 ml/min. The column was eluted at 1 ml/min with a linear 40-min gradient of 0 to 35% 1*M* NaCl in the same buffer; adipsin eluted at 12 to 14% high salt solvent.
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Protection Against Streptococcal Pharyngeal Colonization with a Vaccinia: M Protein Recombinant

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Phagocytosis of group A streptococci requires type-specific antibodies directed against the variable determinants of the bacterial surface M protein molecule. As a step toward developing a broadly protective anti-streptococcal vaccine, a vaccinia virus (VV) recombinant was constructed that expresses the conserved region of the structural gene encoding the M6 molecule (VV:M6′). Mice immunized intranasally with the VV:M6′ virus showed markedly reduced pharyngeal colonization by streptococci after intranasal and oral challenge with these bacteria. M protein–specific serum immunoglobulin G was significantly elevated in vaccinated animals and absent in controls. A similar approach may prove useful for the identification of protective determinants present on other bacterial and viral pathogens.

APPROXIMATELY 25 TO 35 MILLION cases of group A streptococcal infections occur each year in the United States, the most common of which is acute streptococcal pharyngitis in school-age children. Up to 5% of pharyngitis cases that have gone untreated or have been ineffectively treated can lead to acute rheumatic fever, a disease that can ultimately result in cardiac damage. Although this is not a major problem in the United States, except for a recent increase in rheumatic fever cases (*1–3*), this streptococcal sequela is a significant problem in developing nations of the world. By one estimate, nearly 6 million school-age children in India suffer from rheumatic heart disease (*4*).

The ability of the group A streptococcus to cause infection is attributed primarily to the surface-located M protein, an α-helical coiled-coil fibrillar molecule (*5, 6*) that confers to the organism the ability to resist phagocytic attack (*7*). Resistance to streptococcal infection is ascribed to the development of type-specific antibodies directed to the antigenically variable NH_2-terminal determinants of the M molecule (*8, 9*). However, more than 80 antigenically diverse M proteins have been identified, thereby thwarting attempts to use NH_2-terminal M protein epitopes to develop an effective vaccine. An alternative approach, namely the induction of antibodies to epitopes representing the antigenically conserved, surface-exposed COOH-terminal region has proved ineffective in classic mouse virulence models, as these antibodies fail to initiate phagocytosis despite their ability to fix complement as effectively as type-specific antibodies (*9, 10*).

Mice immunized intranasally with the conserved region of the M protein coupled to cholera toxin B–subunit (CTB) showed a significant reduction in mucosal colonization compared with mice receiving CTB alone (*11*). The successful cloning of the streptococcal M protein gene into vaccinia virus (VV) and its expression in viral-infected cells (*12*) has allowed us to investigate the significance of a different antigen delivery system and the protective effects of antibodies directed to the conserved region of the M molecule. In the present study, recombinant VV containing the complete conserved region gene fragment of M6 protein (VV:M6′) (*13*) was used to immunize mice in-

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tranasally. Intranasal and oral challenge with virulent group A streptococci revealed enhanced protection from streptococcal mucosal colonization when compared with protection induced with conserved region peptides linked to CTB (*11*).

A VV vector (VV:M6Δ) has been used to express high concentrations of antigenically authentic M6 protein in a mammalian cell (*12*). The VV:M6Δ recombinant expressed both the variable (type-specific) and conserved portions of the 56-kD M protein. The type-specific NH_2-terminal half of M6 contains the immunodominant epitopes of the M6 molecule (*14*) and induces type-specific protective antibodies to M6 streptococci. Therefore, in an effort to express only the relatively nondominant conserved region epitopes (*14*), we used genetic engineering methods to truncate the NH_2-terminal half of the M6 gene and recombine the COOH-terminal conserved fragment into the VV genome (VV:M6′). This chimeric configuration should allow for the production of a 29-kD truncated M molecule in VV:M6′ infected cells.

We used immunoblot analysis to determine if the VV:M6′ recombinant induced the production of the expected M protein COOH-terminal fragment in mammalian cells and examined whether it is stably expressed. When a COOH-terminal–specific monoclonal antibody was used as a probe, extracts of cells infected with wild-type VV did not reveal any reactive protein, whereas cells infected with the VV:M6′ expressed a protein of an apparent molecular size of 30 kD (Fig. 1). This molecular size is close to the 29-kD expected from the sequence of the cloned M protein fragment (*15*). Thus, like VV virus recombinants containing the complete M6 protein gene (VV:M6Δ) (*12*), a fragment of the M protein consisting of approximately half the native molecule was also expressed in high amounts within viral infected cells (Fig. 1). However, unlike the complete molecule, which is expressed as a double protein band in mammalian cells (*12*) and a triple band in bacterial cells (*16*), the COOH-terminal fragment was expressed as a single homogeneous protein (Fig. 1).

Groups of mice (Swiss CD1) (Jackson Laboratory) were immunized intranasally with either the VV:M6′ virus or the wild-type virus at a dose of 10^7 to 10^8 plaque-forming units (PFU) per mouse. After 4 weeks, mice were challenged both intranasally and orally with M6 group A streptococci (strain S43/192) that were resistant to streptomycin (200 μg/ml). All animals were housed four or five to a cage throughout the experiment. Since group A streptococci are exclusively human pathogens, serial intraperitoneal mouse passage of these organisms is necessary to select for streptococci with the ability to effectively colonize these animals (*7*). However, mouse passage strains have not lost their capacity to infect humans (*7*). Preparation of the strain for challenge was as described (*11*). In two separate challenge experiments, 20 μl of the streptococcal suspension was administered to each nostril (*11*) and 10 μl orally, resulting in a total dose of 6×10^6 to 1×10^7 colony-forming units (CFU) per mouse. At 24 hours after challenge, and at 24- to 72-hour intervals for 10 days thereafter, throats were swabbed (Calgiswab type 4) (Spectrum) and cultured on blood agar plates containing streptomycin (200 μg/ml) (*11*) to facili-

Table 1. Throat cultures for streptococci after intranasal and oral challenge of mice vaccinated intranasally with wild-type or recombinant vaccinia virus. Streptococcal challenge dose in experiment 1 (37 animals) was 6×10^6 CFU and in experiment 2 (16 animals) was 1×10^7 CFU. Colony-forming units of β-hemolytic streptococci on streptomycin-containing blood agar of throat swabs from mice challenged with group A streptococci. D, dead; after autopsy, spleens cultured on streptomycin-containing blood agar revealed β-hemolytic streptococci.

CFU after wild-type VV vaccine							CFU after recombinant VV:M6′ vaccine						
Mice	Days after challenge						Mice	Days after challenge					
	1	2	3	6	8	10		1	2	3	6	8	10
			Experiment 1							*Experiment 1*			
1–3	>100	>100	>100	D			1	0	0	>100	D		
4	>100	92	>100	D			2	0	0	D			
5	63	>100	>100	D			3	0	0	1	>100	D	
6	0	15	D				4	0	5	4	0	3	0
7	43	>100	82	3	4	0	5	0	3	3	1	1	0
8	35	1	17	32	>100	28	6	0	9	0	0	0	0
9	1	0	0	2	>100	5	7	0	1	0	0	0	0
10	0	0	0	>100	>100	>100	8	0	0	0	1	0	0
11	0	0	0	0	65	D	9–20	0	0	0	0	0	0
12	3	8	1	0	1	0				*Experiment 2*			
13	0	0	88	0	0	1	1	>100	>100	D			
14	1	7	0	0	0	0	2	>100	>100	>100	D		
15	4	0	0	0	2	0	3	14	>100	0	50	>100	>100
16	0	0	2	0	0	0	4	0	61	0	0	0	0
17	0	0	2	0	0	0	5	0	3	0	0	0	0
							6–8	0	0	0	0	0	0
			Experiment 2										
1	>100	>100	D										
2	>100	>100	>100	D									
3	>100	>100	13	D									
4	>100	>100	>100	>100	D								
5	36	86	67	D									
6	>100	>100	5	>100	>100	>100							
7	>100	37	2	>100	>100	>100							
8	16	49	32	>100	6	85							
							*Percent culture positive or dead**						
	76	72	76	72	84	76		11	32	25	29	29	21

*Significant difference ($P < 0.005$) at each time point between wild-type and recombinant vaccinated mice by χ^2 analysis. This level of significance is higher than mice immunized intranasally with conserved region peptides linked to CTB (*11*). Whether this difference in protection is related to the method of delivery or to the size of the conserved region will require further experiments.

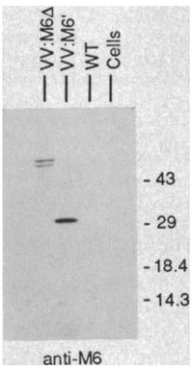

Fig. 1. Immunoblot analysis of the expression of M-specific protein by recombinant vaccinia virus-infected cells. Cytoplasmic extracts were prepared from cells infected for 24 hours with wild-type virus (WT) or VV recombinants containing either the complete M6 gene (VV:M6Δ) or the COOH-terminal conserved region gene fragment (VV:M6′). The extracts were examined by immunoblot with an M protein–specific monoclonal antibody recognizing an epitope in the COOH-terminal half of the M molecule (*20*). An extract from uninfected cells (Cells) was included. Molecular markers: 43 kD, ovalbumin; 29 kD, carbonic anhydrase; 18.4 kD, α-lactoglobin; and 14.3 kD, lysozyme.

Table 2. Kinetic-ELISA analysis of antigen-specific serum and salivary immunoglobulins in mice immunized with VV:M6′ recombinant or wild-type vaccinia. Serum was collected and pooled from four to six mice in each group before and 4 weeks after intranasal immunization and stored at −70°C. Pooled sera were diluted 1:20 and assayed as described (*11, 14*). Whole saliva was collected after stimulation with pilocarpine (*11*) from eight to ten mice in each group before and 4 weeks after immunization (24 hours before challenge). Salivas were pooled, centrifuged at 15,000*g* for 20 min and frozen at −70°C. Pooled salivas were diluted 1:1 and assayed as previously described (*11*). Microtiter wells were sensitized with 100 μl of either purified ColiM6 protein [5 μg/ml; the product of the complete M6 gene expressed in *E. coli* (*16*)], or wild-type VV (5×10^8 PFU/ml) diluted in 10 m*M* phosphate buffer, *p*H 7.4, and processed as described for k-ELISA analysis (*11, 14*). All values are expressed in absorbance per hour.

Vaccine	Antigens			
	Serum (IgG)		Saliva (IgA)	
	M6	Vaccinia	M6	Vaccinia
None	0.35 ± 0.1	1.10 ± 0.5	0.06 ± 0.08	0.02 ± 0.02
VV:M6′	4.60 ± 0.3	6.83 ± 1.3	0.02 ± 0.02	0.29 ± 0.2
WT-VV	0.25 ± 0.2	7.69 ± 0.6	0.04 ± 0.02	0.30 ± 0.1

tate the identification of challenge organisms from normal flora bacteria. Cultures were grown overnight at 37°C and scored for the presence of β-hemolytic streptococci. Mice that had died after challenge were autopsied, and their spleens were aseptically removed and cultured on streptomycin-containing blood agar.

Pharyngeal colonization of animals immunized with wild-type virus differed significantly from those immunized with the VV:M6′ recombinant (Table 1). Of the VV:M6′ vaccinated animals in both experiments, only 16% of the 152 total swabs taken were positive for streptococci, with 10 (6%) yielding >100 CFU, whereas 69% of the 115 swabs were positive in the wild-type group with 40 (35%) displaying >100 CFU. On average, >70% of the 25 animals immunized with wild-type virus were culture positive for group A streptococci at every swab day up to 10 days after challenge (Table 1). This is compared with <30% colonization of 28 mice immunized with the VV:M6′ virus and cultured over the same time period after challenge (Table 1).

In the two challenge experiments, the extent of colonization was comparable within the VV:M6′ and wild-type immunized animals. From 65 to 100% of the animals immunized with the wild-type virus were colonized on day 1 after streptococcal challenge and remained essentially so or died during the duration of the experiment (Table 1). In the VV:M6′ vaccinated mice, 0 of 20 (0%) in experiment 1 and 3 of 8 (38%) in experiment 2 exhibited a positive throat culture on day 1 after challenge (Table 1). Whereas 5 (18%) of the animals died after streptococcal challenge in the VV:M6′ vaccinated group, 12 (48%) died in the wild-type vaccinated animals during the 10 days after challenge (Table 1). Spleen cultures taken of the dead animals revealed β-hemolytic streptococci. These results show that protection against streptococcal colonization in this mouse model follows immunization with the recombinant VV:M6′ virus.

Kinetic enzyme-linked immunosorbent assay (k-ELISA) (*11, 14*) was used to determine the serum immunoglobulin G (IgG) and salivary IgA antibody concentrations to both M protein and VV in immunized mice. At the time of streptococcal challenge (4 weeks after immunization), M protein–specific IgG was significantly elevated in the serum of VV:M6′ vaccinated animals and absent from those animals that received wild-type virus (Table 2). Comparable high concentrations of antibodies to VV were observed in both wild-type and VV:M6′ immunized animals. Whereas low concentrations of IgA directed to VV was found in the whole saliva of animals immunized with either VV:M6′ or wild-type virus, no measurable M protein–specific IgA was identified in the saliva of VV:M6′ immunized animals.

When pooled sera from VV:M6′ or wild-type immunized mice were assayed for the presence of type-specific opsonic antibodies capable of phagocytizing M6 streptococci in whole blood (*8*), they both were found to be negative (*17*). Furthermore, incubating these same pooled antisera with immunoblots containing the NH_2- and COOH-terminal halves of the M6 molecule (*18*) revealed reactivity only with the COOH-terminal conserved fragment with sera from the VV:M6′ immunized mice (*17*). Thus, the observed protection was not the result of induction of type-specific opsonic antibodies.

The incidence of group A streptococcal respiratory infection generally peaks at approximately age 6 and rapidly declines above age 10, reaching adult levels by 18 to 20 years (*19*). Since it is unlikely that during this time exposure to all streptococcal serotypes has occurred, the decreased incidence of streptococcal pharyngitis in adults could be explained by an age-related host factor. Alternatively, since antigenic epitopes in the conserved region of the M molecule are shared among >30 different M proteins (*20–22*), protective non–type-specific immunity may be induced by these common determinants as a consequence of multiple streptococcal infections experienced during childhood. We have found that a vaccinia recombinant expressing the gene encoding the conserved region of the M protein induces an immune response in mice capable of protecting against mucosal colonization after challenge with group A streptococci.

These results confirm and extend previous studies with synthetic peptides from conserved region sequences of the M molecule to induce mucosal protection against streptococcal colonization (*11*). Although animals immunized with VV:M6′ mounted a strong serum IgG response to conserved M protein epitopes, M protein–specific salivary IgA could not be detected by k-ELISA. A very low but measurable M protein–specific IgA response was found in whole saliva from animals immunized intranasally with conserved-region peptides linked to CTB (*11*). This low antigen-specific IgA concentration could (i) be caused by the lack of sensitivity of IgA-specific reagents used in the assay, or (ii) reflect a low but protective concentration of M protein–specific IgA in whole saliva. This latter idea is supported by the fact that conserved region M protein–specific IgA is found in all human saliva examined and M protein–specific IgA is able to protect mice against streptococcal challenge after purification and concentration (*11, 17, 23*). However, this does not rule out the possibility that the observed protection in this mouse model is a result of some other yet undefined immune mechanism induced

by the conserved region vaccine and introduced by this delivery system. It is clear that type-specific antibodies to the M molecule are necessary to protect the host once the streptococcus has initiated infection (*7, 8*). However, our studies reveal that an immune response to the non–type-specific regions will block those events necessary for streptococcal colonization of the mucosal surface in this model system. Although the conserved region used in these studies exhibits >80% sequence identity with known M molecules (*24*), the extent of cross-protection provided by this segment among the known M serotypes remains to be determined.

Whereas the use of VV-based vaccines is not likely to be approved for the control of disease in the near future because of medical concerns regarding safety, the research reported here for the streptococcal M protein provides an example of how such vectors may be used to define the contribution of individual epitopes in the induction of a protective immune response. This approach may be applicable for protection against other pathogenic organisms for which no vaccines now exist, because of problems ascribed to either serotype diversity or antigenic variability of major virulence determinants.

Note added in proof: Experiments were designed to determine if immunization with VV:M6′ is also able to protect mice against colonization after challenge with heterologous streptococcal serotypes. Among 12 mice immunized with VV:M6′ and challenged both intranasally and orally with M14 streptococci (strain T14/46), 17 to 25% exhibited positive throat cultures over a 6-day period while 50 to 70% of 10 animals immunized with either wild-type VV or no virus were colonized by the M14 organisms. By day 6 post-challenge, 20% of the control animals and none of the VV:M6′ immunized animals had died. Although these experiments need to be repeated with this and other streptococcal serotypes, they suggest that immunization with the conserved region of the M6 molecule will significantly reduce colonization by both homologous and heterologous streptococcal serotypes.

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13. The pVV:M6Δ plasmid was cut with Fok I (cleaves at position 751 of the M6 sequence) and Cla I (cleaves at the 3′ end of the insert). The fragment corresponding to the 3′ half of the M6 gene was isolated, the recessed ends filled in with the Klenow fragment of DNA polymerase I, and the blunt-ended fragment cloned into the Sma I site of M13mp19 replicative form DNA. A recombinant phage containing the insert in the correct orientation was identified by hybridization with a strand-specific oligonucleotide probe. Single-stranded DNA was prepared and used as a substrate for oligonucleotide-directed site-specific mutagenesis designed to introduce a G between positions 769 and 770 of the insert. This alteration, which was verified by Sanger dideoxynucleotide sequencing procedures, resulted in the Lys^{209} codon of the M6 open reading frame being converted to an in-frame AGT codon. The mutagenized insert (M6′) was excised from the phage DNA with Eco RI and Bam HI, which cut at the 5′ and 3′ ends, respectively, of the M6′ insert. The recessed ends were blunted with Klenow, and the fragment was blunt-end ligated into the Bam HI site of the pVV3 insertion plasmid, which was also filled in with Klenow. Restriction map and nucleotide sequence procedures were used to select a recombinant plasmid containing the M6′ insert in the correct orientation with regard to the VV 7.5-kD promotor element. This pVV3:M6′ recombination plasmid was used to introduce the chimeric gene into the VV genome by using standard marker transfer technology [C. M. Rice, C. A. Franke, J. H. Strauss, D. E. Hruby, *J. Virol.* **56**, 227 (1985)]. The recombinant virus (VV:M6′) was grown, purified, and then carefully analyzed to verify that the M6′ insert was present, intact, and actively transcribed in virus-infected cells. The genotype of VV:M6′ was apparently quite stable as no deletions or rearrangement of the M protein sequences were evident after multiple passage of the recombinant VV through tissue culture cells.
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Plant Hybrid Zones as Sinks for Pests

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An 8-year study of how aphids are distributed and survive on hybrid and pure host populations showed that the more susceptible hybrid trees acted as pest sinks supporting most of the aphid population. At least 85 to 100 percent of the aphid population was concentrated on less than 3 percent of the host population, with the center of a pest's distribution being the hybrid zone of its host. The concentration of aphids on such a small segment of the host population suggested that susceptible plants not only acted as sinks in ecological time, but may also have prevented aphids from adapting to the more numerous resistant hosts in evolutionary time. This has important implications for the potential management of pest evolution in agriculture and in understanding natural pest distributions.

ALTHOUGH RARELY EXAMINED, THE unparalleled genetic variation that can arise when two plant species hybridize and introgress represents an opportunity to examine how insects and pathogens respond to genetically scrambled hosts (*1*). Since hybridization is known to affect resistance to pests and parasites (*2*), it is of interest to determine how altered host resistance in natural hybrid zones might affect the evolution of plant-pest interactions. Here I examine how a plant parasite, the gall-producing aphid, *Pemphigus betae*, is ecologically and perhaps evolutionarily tied to natural hybrid cottonwoods, *Populus* sp. Most of the study was concentrated along approximately 500 km of the Weber River in northern Utah where Fremont cottonwood, *Populus fremontii*, occupies the lower elevations of riparian habitat and narrowleaf cottonwood, *P. angustifolia*, occupies the upper elevations. A 13-km zone of overlap exists at their common boundary where both species interbreed to produce a hybrid swarm.

The extent of hybridization is demon-

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