Control of Acute Mycoplasmal and Viral Respiratory Tract Disease

The prospects of eventual successful immunoprophylaxis through vaccination are encouraging.

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Over the past 36 years a succession of agents has been etiologically associated with disease of the respiratory tract, and this has led to the current situation in which a majority of the acute respiratory illnesses of man can now be explained as being caused by organisms which are cultivatable in the laboratory (*1*). Starting with influenza A virus, which was first isolated in 1933 (*2*), a veritable cornucopia of respiratory tract pathogens has been uncovered by research microbiologists, providing the basis for ultimate prophylaxis of acute respiratory disease. During the past 16 years the pace has quickened, resulting in an exponential increase in our understanding of the etiology and epidemiology of respiratory illness. First came recognition of the adenoviruses; this was quickly followed by the isolation and characterization of RS virus, the parainfluenza viruses, the rhinoviruses, and, most recently, the coronaviruses (*3*). In 1962, the etiologic agent of cold-agglutinin-positive primary atypical pneumonia was identified as a mycoplasma and given the designation *Mycoplasma pneumoniae* (*4*).

Despite these impressive laboratory, clinical, and epidemiologic achievements, the impact of respiratory viruses and mycoplasmas today is essentially what it was several decades ago. Although the first successful field trial of an inactivated influenza virus vaccine was performed approximately 23 years ago (*5*), influenza viruses continue to ravage the United States and other parts of the world. For example, in 122 cities of the United States, 26,000 excess deaths were attributed to influenza A2 virus in 1957–1958 and 19,500 to Hong Kong influenza A virus in 1968–1969 (*6*). The factors which have limited the effectiveness of licensed commercially produced influenza vaccines—factors which include frequent shifts in the antigenic structure of influenza A virus and the need for yearly injection of vaccine even in the absence of antigenic variation—have been discussed extensively in a number of recent reviews (*7*); for this reason I do not deal with them here in detail.

Vaccines for the noninfluenzal respiratory agents are in various stages of development, but none have been licensed for commercial production. Control of these agents, which cause the major proportion of acute disease of the respiratory tract, promises to be a task more formidable even than the prevention of influenza virus infection.

Difficulties that Impede Progress in Immunoprophylaxis

Multiplicity of agents. The development of effective and practical vaccines for the prevention of respiratory disease is beset with a number of difficulties, the most important being the multiplicity of agents which are responsible for this type of illness. As shown in Table 1, one mycoplasma species and 118 distinct viruses, belonging to seven different virus groups, have been identified as etiologic agents in disease of the human respiratory tract. This multitude of agents poses a problem of unprecedented magnitude for disease control.

Certain viruses, however, are more important than others, particularly when one considers moderate-to-severe disease with involvement of the lower respiratory tract. If effective protection could be provided for 13 agents—influenza A and B viruses; RS virus; parainfluenza virus types 1, 2, and 3; adenovirus types 1, 2, 3, 4, 5, and 7; and *Mycoplasma pneumoniae*—it might be possible to dramatically alter the pattern of serious respiratory disease in man.

Variation in pattern of disease produced by different agents. Many respiratory tract pathogens produce a spectrum of effects in man which range from mild disease of the upper respiratory tract to serious, life-threatening lower tract illness. Certain agents, however, exhibit a greater tendency to produce severe disease than others do. Severe lower respiratory tract involvement occurs most often during RS virus infection of infants and least often during rhinovirus infection of older children and adults. Each agent has a predilection for inducing a particular type of disease, often in a particular host. Such virus-disease associations are summarized in Table 1. In several instances one agent or group of agents may assume major importance in a disease syndrome. For example, RS virus is responsible for the majority of cases of bronchiolitis or pneumonia in young infants, while rhinoviruses are the major definable cause of common-cold-like illness in children and adults (*8, 9*). Furthermore, *Mycoplasma pneumoniae* is the most important cause of pneumonia in older children and young adults (*10*).

Since the pattern of disease and the importance of the various agents varies with age and environmental factors, different vaccine formulations are required to meet these special needs. A formulation suitable for young infants would not be suitable for adults, and vice versa. An effective vaccine for infants should include RS virus; parainfluenza virus types 1, 2, and 3; and adenovirus types 1, 2, 3, 5, and 7. For children the formulation should be expanded to include *Mycoplasma pneumoniae* and the rhinoviruses. Vaccines designed for adults in the general population should include influenza viruses, rhinoviruses, coronaviruses, and *M. pneumoniae*, while the needs of military recruits and other members of semi-isolated, high-density populations would best be met with vaccines for influenza viruses; adenovirus types 3, 4, and 7; and *M. pneumoniae*.

Change in antigenic structure. The influenza A viruses and, to a lesser ex-

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tent, the influenza B viruses undergo progressive change in antigenic structure, older strains or subtypes being replaced with newer antigenic variants (*11*). Fortunately, this type of antigenic drift has not been found in the case of the other respiratory pathogens.

Seroepidemiologic studies suggest that a finite number of antigenic subtypes of influenza A virus exist and that such subtypes may recirculate after a 60- to 70-year interval of quiescence (*12*). If immunoprophylaxis against influenza viruses is to be effective, we must be able to respond rapidly to major antigenic shifts in type A viruses, or anticipate such shifts by providing resistance to the full range of this organism's antigenic diversity.

Change in prevalence. The most extreme example of a pattern of changing prevalence of viruses is provided by the rhinoviruses. These viruses do not appear to undergo a progressive shift in antigenic structure, but they do exhibit a shift in prevalence, in which different serotypes replace each other in an apparently never-ending and unpredictable kaleidoscopic pattern (*9*). If rhinovirus illness is to be controlled, broad antigenic coverage must be provided. This will be a herculean task, since at least 90 distinct rhinovirus serotypes are now known to exist.

Transient resistance and reinfection. Certain respiratory viruses are able to infect and at times produce disease even though the host has been previously infected with the agent and has a moderately high concentration of serum antibody (*13, 14*). The most extreme example of this type of behavior is exhibited by RS virus. In large urban populations this agent causes an epidemic each year, in which reinfection of children and adults is a common occurrence; these individuals then serve as the source of virus for infection of the young infant, who is the host in whom the most severe disease manifestations develop (*15*). In studies with volunteers we have been amazed by the ease with which RS virus reinfects adults who have high concentrations of naturally acquired neutralizing antibodies in the serum and in nasal secretions (*16*). Nevertheless, the most severe illnesses caused by this virus occur during primary infection.

Complete resistance to infection with parainfluenza virus also appears to be evanescent, although, as in the case of RS virus, the most severe illness generally occurs during first infection (*13*).

Table 1. Viruses and mycoplasmas which cause respiratory disease in man.

Group of agents	Serotypes		Most important clinical consequences of infection
	No.	No. associated with respiratory illness	
Myxovirus			
Influenza	3	2	Influenzal disease in all age groups; pneumonia in adults
Parainfluenza	4	4	Croup in infants and children; also upper or lower tract disease in this age group
RS	1	1	Bronchiolitis and/or pneumonia in infants; lower tract disease in children
Coronavirus	3 or more	3	Common colds in adults
Picornavirus			
Coxsackie A and B	29	10*	Febrile pharyngitis in infants and children; colds in military recruits (A-21)
Rhinovirus	90 or more	90 or more	Common colds in children and adults
Adenovirus	30	8†	Upper or lower tract disease in infants and children; same in military recruits
Mycoplasma	9	1‡	Pneumonia in children and young adults

* Coxsackie A2, 4, 5, 6, 8, 10, and 21; Coxsackie B2, 3, and 5. † Adenovirus types 1, 2, 3, 4, 5, 7, 14, and 21. ‡ *Mycoplasma pneumoniae.*

Effective resistance to the disease-producing effects of an antigenically stable influenza A virus may not extend beyond 2 years (*17*). The most durable immunity appears to be associated with the pathogenic adenoviruses (types 1 to 5, and 7), which rarely cause reinfection and disease in adults (*18*).

The short-lived resistance to infection and illness which follows extensive replication of certain agents in the respiratory tract poses a special problem for the development of effective vaccines. Clearly, at the very least, the resistance produced by natural infection must be matched by vaccines. Ideally, a more durable immunity would be desirable. Viruses such as RS and the parainfluenza viruses, which reinfect readily, will be difficult to eradicate from human populations, and the goal of producing vaccines for these viruses must necessarily be limited to the prevention of disease.

Contribution of host immunologic factors to disease. In at least two settings, host immunologic factors appear to play as large a role in pathogenesis of disease as the direct effects of the virus itself. RS virus disease of the lower respiratory tract in young infants and the altered, exaggerated response to RS virus infection exhibited by recipients of inactivated parenterally administered RS vaccine represent reactions in which the host contributes as much to the equation of illness as the virus does (*19*). My associates and I have postulated that both of these severe types of reaction to RS virus infection represent a marked enhancement of the basic virulence of the organism through an interaction of viral antigens and serum antibodies in the lungs of individuals who lack protective amounts of secretory antibody in the respiratory tract (*19*). In the natural disease of young infants this interaction may occur because the infant, though it possesses maternally transmitted serum antibodies, lacks local respiratory tract immunity. Similarly, infants injected with a potent inactivated vaccine developed serum antibodies but no effective local respiratory tract immunity. Other workers (*20*) have suggested that vaccine-altered reactivity to RS virus infection is the result of cell-mediated immunity (delayed hypersensitivity) induced by the vaccine. For reasons given elsewhere (*19*), we favor a unitary hypothesis which links both the natural illness of young infants and vaccine-induced altered reactivity to a reaction of RS virus antigen and serum antibody which occurs in the lungs.

At present the relevance of immunologically mediated RS virus disease to other respiratory illnesses is not clear, but this is a new area and it is not unlikely that additional situations resembling that seen with RS virus will become evident. Nonetheless, the importance of immunologic factors in RS virus disease of infancy and in illness potentiated by RS vaccine adds a new dimension to the problems of experimental immunoprophylaxis. In efforts to vaccinate against RS virus, and against other viruses as well, great care must be taken to stimulate the type of immune response which provides resist-

ance against infection and illness and, at the same time, avoid selective induction of that type of immune response which contributes to the development of disease.

Safety. At present, our greatest area of concern centers on the question of the safety of the vaccines. With the exception of illnesses caused by RS and influenza viruses, the diseases we are attempting to prevent have a negligible mortality rate. This means that vaccines for respiratory tract disease designed for widespread use must be essentially free of risk to the recipient. The known hazards can be avoided, but it is always difficult to predict and assess risks on the basis of analogy. The large number of identifiable adventitious agents which contaminate tissue culture cells used for the production of vaccines do not pose a serious problem, since the technology for detecting these organisms has been developed to an advanced state. This subject is discussed in an excellent recent review by Meyer (*21*).

In my view, there are two major causes for concern. The first involves the vaccine virus itself. Parenterally administered inactivated antigens of RS virus and measles virus have been shown to induce a state of altered reactivity to subsequent naturally acquired infection. Now that we have become "sensitized" to the sensitizing potential of these and possibly other viruses, it is unlikely that this type of adverse vaccine effect will be seen in the future.

The vaccine virus may pose a threat which is more ominous than sensitization—namely, the threat of oncogenicity. This possibility was first raised when it was found that several human adenovirus serotypes induced sarcomas when injected into newborn hamsters (*22*). However, as discussed below, there is increasing evidence that these viruses are not related to human cancer.

The second major cause for concern pertains to the RNA "C type" leucosis viruses, which are commonly present in cells of various animal species (*23*). Unfortunately, infection may be covert, and the virus genome (or portion of the genome) may be present in a form which eludes detection by the most sensitive assay system (*23*). In such circumstances our familiarity with the safety of materials such as beef, milk, and eggs may be more reassuring than negative results in tests for RNA leucosis viruses in bovine or avian tissue cultures used for producing vaccines. It should not be forgotten that a continuous safety test of calf lymph has been in progress for 170 years, since Jenner first introduced vaccination against smallpox.

Immunologic Determinants of Resistance to Infection and Illness

Critical to the development of effective vaccines for the control of acute disease of the respiratory tract is an understanding of the immunological factors that provide protection against the important respiratory pathogens. There seems little reason to doubt that antibody in serum is a prime mediator of immunity for viruses which undergo a systemic phase of dissemination. Until recently, it was commonly thought that antibody in serum was also a prime mediator of immunity for infections localized in the respiratory tract. The first doubts were raised in the early 1940's when Francis (*24*) detected neutralizing activity for influenza virus in nasal secretions and suggested that local defense mechanisms might play a significant role in the prevention of influenza. Subsequently these doubts were amplified by the elegant studies of Fazekas de St. Groth, almost 20 years ago (*25*). He showed that resistance to influenza A virus infection experimentally induced in the mouse was mediated primarily by antibody present in respiratory tract secretions, while antibody in serum was relatively ineffective.

The finding that antibody in respiratory tract secretions plays a major role in resistance to influenza in the mouse is not surprising when one considers the superficial nature of this myxovirus infection. Local antibody is in the right place to intercept virus which attacks the respiratory tract epithelium. At the time Fazekas defined the role of local antibody in experimental murine influenza, the existence of different immunoglobulin classes was not known. In the ensuing two decades there have been rapid advances in our understanding of the structure and sites of synthesis of immunoglobulins. It is now clear that the major functionally active immunoglobulin (Ig) in respiratory tract secretions is a dimeric 11*S* IgA, whereas most serum antibodies which are active against respiratory viruses are of the 7*S* IgG variety (*26*). Furthermore, secretory IgA antibody appears to be synthesized locally in plasma cells located beneath the mucosal epithelium. Thus, the secretory antibody system is distinct from the serum antibody system both in site of synthesis and in type of immunoglobulin produced.

In man, local respiratory tract antibody appears to be of major importance in providing resistance to type 1 parainfluenza virus, RS virus, and rhinovirus infections. Some of the experimental and epidemiological data which support this view are described here because of the significance of these observations to the direction of vaccine development.

Type 1 parainfluenza virus. Shortly after the initial isolation of type 1 parainfluenza virus in our laboratory we experimentally challenged volunteers with the virus to determine the type of illness it produced in adults (*27*). We had shown that type 1 virus was a major cause of severe croup in young children, but we lacked information about its behavior in adults (*28*). To our surprise, about two-thirds of the volunteers became infected and developed common-cold-like illness, although the dose of virus given was small (80 $TCID_{50}$) and most of the men had moderate concentrations of serum neutralizing antibody prior to challenge. There was no apparent relationship between level of serum antibody and infection of the volunteers (*27*). In a subsequent study with volunteers this situation was clarified when it was shown that nasal secretion 11*S* IgA antibody was closely related to resistance, whereas serum antibody appeared to be without effect (*29*).

RS virus. RS virus is unique in that it causes severe disease most often during the first half year of life (*15*). This pattern provides an unusual opportunity to assess the protective effect of serum antibody for this virus in the absence of any potential effect of local respiratory tract immunity. Every infant is born with a moderately high level of passively acquired serum neutralizing antibody, and this antibody is present at a detectable level until the 6th to 8th month of life (*19*). Since serious RS virus disease occurs most often in young infants who have high levels of maternally derived antibody, presumably IgG, it is apparent that serum antibody does not protect against infection or its serious effects (*19*).

Children and adults reinfected with RS virus develop less serious illness than infants undergoing primary infection. Since this difference in response did not appear to be related to serum antibody, we turned our attention to the potential role of 11*S* IgA respiratory tract secretory antibody in resistance. In a recent study with volunteers, 16 men were selected for nasopharyngeal challenge with RS virus (500 plaque-forming units per milliliter). Eight of the men (group 1) had a low concentration of nasal secretion antibody, the other eight (group 2) had a high concentration; there was no overlap in the concentrations of secretory antibody between the two groups (*16*). Each of the volunteers had a moderately high concentration of serum neutralizing antibody. Following challenge, each of the men in the two groups shed virus, and the temporal pattern of shedding was the same for the two groups. However, when the virus content of the nasopharyngeal washings was estimated by means of the plaque technique, a marked difference was found in the quantities of virus shed. The group-1 men shed large quantities of virus (up to 10^5 plaque-forming units per milliliter of nasopharyngeal washing), whereas the washings of the group-2 men contained little virus. Following infection, six of the eight group-1 men showed a rise in serum antibody or nasal secretion antibody, or both, whereas none of the group-2 men exhibited such a response. Although the concentration of nasal secretion antibody did not influence susceptibility to challenge with the RS virus, this antibody appeared to have a marked effect upon the degree of virus replication and, secondarily, upon the immune response to infection.

Rhinovirus. Infection with rhinoviruses, as well as with other respiratory viruses, generally induces the development of both local respiratory tract antibodies and serum antibodies. Thus, individuals who have undergone infection often possess both types of antibody, and there is a rough correlation in the levels of these antibodies. It was not surprising, therefore, to find a correlation between levels of both types of antibody and resistance to rhinovirus illness in volunteers, and to find that efforts to assess the importance of either antibody by itself were unsuccessful (*30*). Recently Perkins attempted to dissociate the effects of the two antibody systems by selectively stimulating the production of nasal secretion antibody by intranasal instillation of inactivated rhinovirus type 13 vaccine (*30*). When volunteers who lacked serum antibody ("seronegative" volunteers) were vaccinated in this manner, most of them developed local respiratory tract antibody, and, much to our surprise, almost all of them developed moderately high levels of serum neutralizing antibody (*30*). Ultimately, the desired information regarding the relative significance of serum antibodies and nasal secretion antibodies was obtained by comparing the protection afforded by intramuscular administration of inactivated type 13 vaccine (which stimulated primarily a serum antibody response) with the protection provided by intranasal instillation of vaccine (which stimulated the development of both serum and nasal antibodies). Vaccine given intramuscularly was more effective in stimulating development of serum antibody (mean titer, 1 : 72.5) than nasal administration of antigen was (mean titer, 1:53.8). In contrast, nasal secretion antibody was most effectively induced by nasal vaccination. When the two groups of vaccinated subjects were challenged with type 13 virus (100 $TCID_{50}$), significant resistance to infection and illness was observed in the group given vaccine intranasally, whereas the response of the group given vaccine intramuscularly did not differ from that of a group of unvaccinated seronegative controls (*31*). This pattern of response suggested that nasal secretion antibody was the prime determinant of resistance to rhinovirus illness.

In the three situations just discussed, local respiratory tract antibody was closely correlated with resistance to myxovirus and rhinovirus infection. At this point it is tempting to conclude that this antibody was responsible for the resistance observed in these studies. However, some caution is necessary, since local antibody may only correlate with another local immune mechanism which is the true determinant of protection. In the past, serum antibody was assumed to be the immunologic mediator of resistance to myxovirus and rhinovirus infections, but it now seems likely that the apparent role of serum antibody was only a reflection of the antibody's correlation with local respiratory tract antibody in individuals who had undergone infection. Even if local antibody should subsequently be shown to be an indirect barometer of resistance, this antibody is nevertheless significant, since it is a relatively reliable index of host resistance and thus a valuable guidepost in both epidemiologic studies and vaccine trials.

Adenovirus. Resistance to adenovirus infection differs from the situation for myxoviruses and rhinoviruses. Recent experience with a live type 4 adenovirus vaccine administered in an enteric coated capsule or tablet suggests that serum antibody provides definite protection against adenovirus infection and illness. When type 4 adenovirus is administered in this way, the virus produces a silent infection which is limited to the lower intestinal tract (*32*). Individuals infected by this method develop moderately high levels of serum neutralizing antibody, but, as was recently shown by Smith and his collaborators at the Walter Reed Army Institute of Research, nasal secretion antibody is not induced (*33*). Military recruits who receive the type 4 adenovirus vaccine in an enteric coated capsule prior to an epidemic exhibit significant resistance to infection and almost complete protection against disease caused by this virus (*34*). In this setting, in which a dissociation of serum antibody and local respiratory tract antibody is achieved, it appears that serum antibody by itself is sufficient to provide protection against adenovirus. This finding suggests that type 4 adenovirus produces a type of infection different from that produced by the myxoviruses or rhinoviruses. It may be that adenovirus infection penetrates to a deeper level than the more superficial myxoviruses and rhinoviruses. Certain features of adenovirus infection, such as latency, prolonged shedding, and lymph node involvement, are consistent with this view.

Evaluation of Vaccines for Rhinoviruses and Myxoviruses

Implications of local respiratory tract antibody. Heretofore, experimental vaccines for respiratory illness have been evaluated for antigenicity primarily on the basis of their capacity to stimulate development of serum antibody in animals or volunteer subjects. Since local respiratory tract antibody provides a more meaningful index of host resist-

ance to myxoviruses and rhinoviruses, it seems appropriate to focus our attention upon this type of antibody when evaluating vaccines for these viruses. It also follows that primary consideration should be given to the development of myxovirus and rhinovirus vaccines which stimulate the highest and most lasting respiratory tract secretory antibody response. This view is supported by two examples from our recent experience with experimental vaccines.

1) Several years ago we compared the protective effect of (i) experimental infection with type 1 parainfluenza virus and (ii) parenteral administration of inactivated type 1 virus vaccine (*29*). Eighty percent of the adult volunteers who received inactivated vaccine developed a fourfold, or greater, increase in serum neutralizing antibody, and the mean serum titer was higher after vaccination than after infection. In contrast, the experimentally infected volunteers developed antibody in their nasal secretions more often, and to a higher mean titer, than the vaccinated subjects did. The parenterally injected vaccine evidently did not induce resistance to infection, since six of nine vaccinated subjects who were challenged with live virus became infected; this response to challenge did not differ from that of unvaccinated control subjects. In contrast, infection did not occur in 23 challenges involving volunteers who had been experimentally infected previously and who possessed antibody in their nasal secretions. In this study we observed a direct correlation between induction of nasal secretion antibody and resistance to experimental infection (*29*).

2) As described above, an inactivated type 13 rhinovirus vaccine given intranasally induced resistance to illness experimentally produced with type 13 virus, whereas protection was not observed following intramuscular administration of vaccine (*31*). Topical application of vaccine stimulated development of both serum antibodies and nasal secretion antibodies, whereas parenteral administration of vaccine induced primarily a serum antibody response which was slightly higher than that seen after intranasal vaccination. As in the trial with type 1 parainfluenza virus vaccine, resistance was associated with the development of antibody in nasal secretions.

If respiratory tract antibody plays such a decisive role in resistance, why have parenterally administered inactivated influenza virus vaccines provided protection in the past? The answer may lie in the observation that the respiratory tract secretory antibody mechanism can be stimulated by parenterally administered antigen. We have seen this happen, on occasion, following intramuscular inoculation of rhinovirus type 13 or parainfluenza type 1 virus vaccine (*29*, *31*). Others have described similar findings with antigens of salmonella or influenza A virus, and the local antibody which developed was shown to be of the IgA variety (*35*, *36*). Transport of antigen or sensitized cells from the site of inoculation to the respiratory tract seems a likely explanation for this phenomenon.

Although parenterally administered antigen can stimulate the development of local respiratory tract antibody, this type of immune response can be induced more efficiently and more frequently by direct introduction of either live or inactivated antigen into the nasopharynx. The superiority of direct introduction of antigen was seen during our studies with parainfluenza type 1 virus and rhinovirus vaccine, and it has been demonstrated convincingly by Kasel and Waldman and their associates with influenza virus antigens (*36*).

Doubtless, parenteral vaccines which provide some protection against illnesses caused by parainfluenza virus and rhinovirus can be developed, but the effort and expense involved in their preparation and the need for repeated injections might well outweigh the potential benefits. A case in point is the trivalent parainfluenza virus vaccine recently described by workers at the Merck Institute (*37*). This inactivated vaccine was highly antigenic in animals and stimulated the development of moderately high levels of serum antibody in infants and children. However, a 66-fold concentration of infected monkey kidney tissue culture harvests and addition of alum adjuvant were necessary in order to produce this impressive antigenic activity.

The use of a potent adjuvant, such as peanut oil, has been advocated as a means of potentiating and prolonging the host's humoral antibody response and decreasing the requirement for antigen (*38*). There is no question that this approach produces a higher and more sustained serum antibody response, but the data regarding protective effect are not quite so impressive. In a recent study it was shown that, after 2 years, the protective effect of an influenza A2 vaccine with a potent peanut oil adjuvant was only 55 percent (*39*).

Our most pressing need at this time is not for conventional parenteral adjuvants but for materials which will enhance and prolong the production of local respiratory tract antibody. Little is known about the dynamics of this local antibody system, and there is essentially no information available concerning factors or substances which might potentiate its response.

Experiences with Recently Developed Experimental Vaccines

RS virus. The RS virus is the most important respiratory tract pathogen of early life, and for this reason it has been given one of the highest priorities in vaccine development. After several early, relatively unconcentrated, inactivated vaccines were found to be weakly antigenic in children, a concentrated (100-fold) alum-adsorbed vaccine was prepared from virus grown in vervet monkey kidney tissue culture, in an effort to determine whether high levels of antibody could be stimulated in infants, and whether such antibody would protect against RS virus disease (*14*, *20*, *40*, *41*). The vaccine was prepared and tested before the importance of immunologic factors in the pathogenesis of RS virsus disease was appreciated. In fact, the results of the vaccine trials were instrumental in bringing the role of immunologic factors in RS virus disease into focus.

The vaccine was quite antigenic and stimulated serum neutralizing antibody titers of 1:1000 to 1:10,000 or more in seronegative infants (*14*). However, in four separate studies the vaccine did not appear to protect against RS virus infection; during periods when RS virus was prevalent, the virus was recovered from vaccinated subjects almost as often as it was recovered from members of the control groups (*14*, *20*, *40*, *41*). More striking than the failure of the vaccine to protect against infection was the unexpected response to infection exhibited by vaccinated infants: they developed serious obstructive lower respiratory tract disease at an unusually high rate (*14*, *20*). Clearly, the vaccine had induced an altered and exaggerated state of reactivity to infection, but this effect was limited to the younger subjects who lacked pro-

tective local respiratory tract immunity (*14*). At present we interpret this untoward effect of vaccination as resulting from a type II (cytotoxic) or type III (extracellular Arthus reaction) immunologic reaction produced in the lung by interaction of vaccine-induced serum antibody and RS virus antigen (*19*).

The experience with the inactivated vaccine indicated the need for another type of vaccine which would effectively stimulate the development of local respiratory tract antibody. We have attempted to meet this need by developing an attenuated virus vaccine which can be administered directly into the nasopharynx. The A2 strain of RS virus was attenuated, for man, by successive passage in bovine embryonic kidney tissue culture at reduced temperature (28° or 26°C) (*42*). After 52 passages, the last 16 at 26°C, the virus exhibited both decreased infectivity and lack of virulence when tested in 45 adult volunteers (*42*).

Kim and Parrott, at Children's Hospital of the District of Columbia, have given this potential attenuated vaccine virus to 34 children ranging in age from 2 to 13 years (*43*). Included in this group were ten children 2 to 4 years old. Sixty-one percent of the children were infected when approximately $10^{4.0}$ $TCID_{50}$ of virus was instilled into the nasopharynx (*43*). None of these infections was associated with illness of any sort. Twelve of the 21 children infected developed a significant rise in nasal secretion neutralizing antibody (*43*). The results of the current study are encouraging, and the virus grown at 26°C appears to be completely benign. Possibly this virus will prove to be too attenuated to be useful in the prevention of disease in young infants, the group in greatest need of protection. Since each of the children who received the vaccine virus had had prior experience with RS virus, the critical question of the virulence of the low-temperature strain for previously uninfected infants, including infants with passively acquired serum antibody, remains to be answered.

Parainfluenza viruses. Antigenic inactivated vaccines have been prepared for the parainfluenza viruses, second in importance only to RS virus as respiratory pathogens in early life. Although a trivalent vaccine containing egg-grown viruses induced appreciable concentrations of serum antibody in infants and young children, this preparation did not provide protection against naturally occurring parainfluenza virus disease in two separate trials (*20, 41*). The more concentrated Merck Institute vaccine stimulated the development of somewhat higher concentrations of serum antibody, but unfortunately the rate of parainfluenza virus infection in the study population was not great enough to permit unequivocal evaluation of the vaccine's protective effect (*37*).

Rhinoviruses. As described above, intranasal instillation of an unconcentrated, inactivated type 13 rhinovirus vaccine induced both nasal secretion antibodies and serum antibodies and produced significant resistance to experimentally induced illness. This mode of administering vaccine is simple, and the approach could conceivably be expanded to provide protection against the multitude of serotypes which, together, cause most of the common cold illnesses in older children and adults. This multitude of serotypes constitutes the major obstacle to the success of the intranasal vaccine approach. In addition, techniques for producing higher yields of rhinovirus antigens in tissue culture and methods for potentiating and prolonging the local respiratory tract antibody response must be developed if this type of vaccination is to succeed.

Adenoviruses. The adenoviruses produce their most dramatic effect in semiclosed populations of military recruits, where type 4 virus and, to a lesser extent, type 3 and type 7 virus are major causes of epidemic respiratory tract disease (*18*). Less dramatic, but probably more important in terms of total morbidity, is the contribution of virus types 1, 2, 3, 5, and 7 to acute respiratory disease of infancy and childhood (*44*).

Progress in the development of effective adenovirus vaccines has been slow, primarily because of the fear that these viruses, or their genetic material, might be oncogenic for man. A number of the adenovirus serotypes have been shown to induce tumor formation on being injected into suckling hamsters, but attempts to link these viruses to tumors in man have been uniformly unsuccessful (*45, 46*). An extensive and well-controlled evaluation of adenoviruses in human tumors was recently completed by a collaborative group of the Special Virus Cancer Program of the National Cancer Institute, Bethesda, Maryland. This group of workers was unable to detect antibody for virus-induced, nonvirion, adenovirus T antigens in the serums of patients with various types of cancer (*45*). In addition, Green has examined 150 human tumors for the presence of adenovirus-specific messenger RNA. He was unable to detect such RNA in a single tumor preparation, although the techniques used were sufficiently sensitive to detect 1/10 the quantity of adenovirus-directed RNA that is regularly produced in rat or hamster cells transformed by viruses of the adenovirus group (*46*). These results suggest that adenoviruses probably do not cause tumors in man, since the laboratory methods used were those which firmly link these viruses to the tumors they induce in laboratory rodents.

Although effective vaccines are needed for at least eight adenovirus serotypes (types 1, 2, 3, 4, 5, 7, 14, and 21), we have restricted our efforts to the development and evaluation of a vaccine for type 4 virus. This virus causes large-scale epidemics of acute respiratory disease in military recruits, and this epidemiologic situation offers an ideal setting in which to evaluate the efficacy of a vaccine (*34*). Furthermore, type 4 virus lacks oncogenic potential for the newborn hamster (*32*).

In our studies, type 4 virus was used as a model, with the expectation that the enteric approach to vaccination could be applied to the other adenovirus serotypes of importance in human disease when the specter of oncogenesis has been completely dispelled. Approximately 6 years ago we administered type 4 virus by an atypical route so as to bypass the region of the body in which disease manifestations usually develop (*47*). When type 4 virus was placed in an enteric coated capsule or tablet and fed to adult volunteers, a selective infection of the lower intestinal tract occurred. In this manner the upper respiratory tract was bypassed and infection was confined to the lower intestinal tract. This type of infection was asymptomatic and stimulated the development of moderately high levels of serum neutralizing antibody. Finally, infection did not spread from enterically infected prisoner volunteers or military recruits to susceptible individuals, despite close and prolonged exposure (*32, 34*).

In subsequent studies, Jackson and his associates found that enteric infection was transmitted in some instances

from husband to wife or vice versa; however, secondary infection did not result in respiratory tract disease (*48*). Although, on occasion, transmission may occur between marital partners, it seems clear that contact infection is not a risk in closely associated groups of military recruits (*34*).

To date, enteric coated type 4 virus, grown in human diploid fibroblast culture, has been given to several hundred thousand military recruits without evidence of untoward effect. The vaccine has been highly effective in preventing acute respiratory tract disease caused by type 4 virus. If vaccine is given prior to an epidemic, essentially complete protection against febrile disease caused by type 4 virus is provided (*34*).

In several military recruit centers, suppression of type 4 virus by vaccination has led to the emergence of type 7 virus as a cause of epidemic disease (*49*). Currently, Buescher, Top, and their associates at the Walter Reed Army Institute of Research are evaluating an experimental type 7 enteric virus formulation for use in preventing resurgent type 7 disease in military recruits. In our initial studies we found that type 7 virus behaved like type 4 virus; enteric administration of type 7 virus led to a silent selective intestinal infection which did not spread to individuals in close contact with the infected subject (*47*). In addition, type 4 and type 7 viruses could be administered simultaneously by the enteric route without interference occurring.

An alternative and equally worthy approach to adenovirus immunoprophylaxis involves the parenteral administration of purified, DNA-free, protein subunits of the virus. Partially purified subunit preparations of several adenovirus serotypes were evaluated by Kasel and his co-workers and were found to be moderately antigenic (*50*). Commerical techniques for large-scale production of a partially purified subunit preparation of type 5 adenovirus have been developed by Metzgar and his associates, and one lot of vaccine prepared by this method has proved to be antigenic in seronegative adult volunteers (*51*). More advanced methods for rendering subunit preparations almost completely free of viral DNA are now available, as a result of the efforts of Neurath and Rubin, and will shortly be applied to the production of experimental vaccine (*52*).

The use of parenteral inactivated vaccine represents a reasonable approach to adenovirus immunoprophylaxis, since studies with the enteric vaccine have shown that serum antibody by itself is capable of providing resistance to the disease manifestations of type 4 virus. Whether the live enteric vaccine approach or the purified subunit vaccine approach will be ultimately favored is difficult to predict at this time. It is likely, however, that the balance will shift toward enteric live vaccines as concern regarding the danger of oncogenesis recedes.

Mycoplasma. The need for an effective *Mycoplasma pneumoniae* vaccine is indicated by (i) the incidence of disease in children and in young adults, particularly those in military training, (ii) the frequent prolonged course of undiagnosed disease, and (iii) the failure of tetracycline or erythromycin to eradicate the organism from the pharynx. Initially the development of an inactivated vaccine was hindered by the poor growth of *M. pneumoniae* and the presence of sensitizing components such as horse serum and beef heart infusion in the growth medium. These problems were solved by employing a well-adapted strain of *M. pneumoniae* which grew to high titer in artificial medium in which chemically defined tissue culture media and a chloroform extract of egg yolk were substituted for beef heart infusion and horse serum (*53*). A Formalin-inactivated vaccine was prepared from organisms grown in this medium and concentrated by centrifugation. This vaccine, injected intramuscularly, stimulated the development of growth-inhibiting antibody in ten of 19 (53 percent) seronegative volunteers (*54*). When these and 13 additional seronegative volunteers were challenged with approximately 10^6 colony-forming units of a virulent strain of *M. pneumoniae,* each of the men was infected; however, only one of the ten volunteers with vaccine-induced antibody became ill, whereas ten of the 13 seronegative unvaccinated men developed definite respiratory tract disease. This difference in response to challenge indicated that vaccine-induced serum antibody was associated with resistance (*54*). Whether this resistance was a function of serum antibody or a manifestation of a relationship of serum antibody to another immune system (local respiratory tract antibody or cell-mediated immunity?) remains to be determined. Vaccinated individuals who did not develop detectable serum growth-inhibiting antibody developed more severe disease following challenge than the unvaccinated controls did. At present the basis for this apparent sensitization is not understood, but the possible occurrence of this phenomenon must be taken into account when inactivated mycoplasma vaccine is given.

Evaluation, in a study with military recruits, of an inactivated vaccine prepared with chloroform extract of egg yolk indicated that immunization provided measurable but not complete resistance. A 46 percent reduction in *Mycoplasma pneumoniae*–associated pneumonia was observed, and there was no evidence of disease potentiation (*55*). Although a definite protective effect of the vaccine was demonstrated, this preparation was not of sufficient potency to be considered suitable for widespread use. The importance of the study lay in the finding that inactivated vaccine was capable of inducing resistance to naturally occurring illness produced by *M. pneumoniae.*

Another inactivated vaccine has been described in which *Mycoplasma pneumoniae* was grown in a "serum free" medium (of unidentified composition) in order to diminish unwanted antigenic medium components (*56*). When administered with an alum adjuvant, the vaccine effected a 45 percent reduction of pneumonia in a population of approximately 21,000 military recruits studied by Mogabgab (*56*). It is difficult to compare the protective effect of this vaccine and that grown in egg yolk medium, but it appears that the two vaccines are of about equal potency. The potency is low, but they are, nevertheless, prototype vaccines and they demonstrate that vaccine-induced protection from *M. pneumoniae* illness is feasible.

My associates and I have described a new technique for the culivation of *Mycoplasma pneumoniae* which offers some promise for the development of more potent inactivated vaccines. Under specific conditions the organism grows luxuriantly on glass and remains tenaciously attached to the glass surface despite repeated washings with saline solution or water (*57*). This property makes it possible to remove medium constituents from the organisms; the organisms can then be scraped from the glass surface, to yield a highly concentrated purified suspension of mycoplasmas suitable for use in the production of vaccine. The *p*H must be maintained at or near neutral-

ity, since a shift in *p*H into the acid range produces a decrease in the antigenicity of the glass-grown organisms (*58*).

The experimental hamster studies of Fernald suggest that prior infection is more effective than parenterally administered inactivated vaccine in stimulating resistance to *Mycoplasma pneumoniae* (*59*). Unfortunately, attempts to develop a satisfactory attenuated live vaccine have thus far been unsuccessful. Although we found that serial passage of the organism in mycoplasma broth medium resulted in attenuation of virulence for man, this attenuation was accompanied by a decrease in infectivity (*60*). In addition, these attenuated strains of *M. pneumoniae* exhibited a degree of residual virulence which made them unsuitable for use in a live vaccine.

Uses of Genetic Techniques for Development of Vaccines

Because of the overriding importance of local respiratory tract immunity in resistance to respiratory pathogens, we have continued to search for new approaches to the development of attenuated mutants which could be used in live vaccines. The most promising new approach currently under study is based in part upon the temperature differential which exists in the respiratory tract: the temperature of the nasal mucosa is 32° to 34°C, while the temperature of the lower tract is 37°C. We sought to take advantage of this temperature differential and select virus or mycoplasma mutants which would grow vigorously at 32° to 34°C but which would not replicate efficiently at 37° to 39°C. Theoretically, mutants of this type would not be able to produce disease in the lower respiratory tract because of their failure to grow to high titer in this location.

Mutants were produced by exposing virus to either 5-fluorouridine or 5-fluorouracil during viral replication, or by exposing resting *Mycoplasma pneumoniae* organisms to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Subsequently, the desired mutants were sought in populations of "mutagenized" virus or mycoplasma by testing the progeny of single infectious units for plaque- or colony-forming efficiency at 32°C, which was selected as the permissive temperature (the temperature at which growth should occur) and 38° or 39°C, the restrictive temperature (the temperature at which growth should be suppressed). Those plaque or colony isolates from a mutagen-treated population of organisms which produced virus plaques or mycoplasma colonies at the permissive temperature, but not at the restrictive temperature, represented the desired conditional lethal temperature sensitive (ts) mutants.

Until now, ts mutants have been used primarily for mapping the genome of viruses and for delineating the genetic basis for certain biologic properties of the organism under study (*61*). Such mutants are useful for these purposes since almost any gene can be affected by a ts mutation, the capacity of mutants to grow is dependent upon environmental conditions, and the extent of the temperature limitation can be imposed by the selection procedure used by the investigator (*61*). These properties of the ts class of mutants proved to be equally advantageous in our studies.

RS virus. Gharpure and Wright detected four ts mutants of RS virus among 454 clones derived from virus grown in bovine kidney tissue culture in the presence of $10^{-4}M$ 5-fluorouridine (*62*). The mutants were stable, with reversion frequencies of less than $10^{-5.0}$. Their capacity to initiate foci of infection (that is, to produce plaques) in tissue culture was markedly or completely suppressed at 37°C (*62*). The temperature-sensitive defect of three of the mutants appeared to affect functions which were expressed late in the replicative cycle. One of the mutants produced atypical nonsyncytial plaques which made it possible to score this mutant in the presence of the other mutants. Complementation analysis indicated that three of the mutants had defects which affected the same cistron, while the fourth mutant (which produced atypical plaques) was temperature-sensitive because of a defect in another cistron (*63*).

The parent virus and the first of the ts mutants (ts-1) were compared for in vivo growth properties in 3-week-old hamsters. Unlike the parent virus, which grew in both the nasal turbinates and the lungs, growth of the mutant was confined to the upper respiratory passages. These encouraging findings indicate that restriction of growth at 37°C also occurs in vivo. Infection of hamsters with the ts-1 mutant or the ts-4 mutant stimulated a moderately high serum complement fixation and neutralizing antibody response and induced significant resistance to subsequent challenge with unmodified parent virus (*63*).

Influenza A virus. In a study performed by Mills and VanKirk (*64*), two ts mutants of influenza A_2 (1965) virus were produced by growth of the agent in bovine kidney tissue culture in the presence of $10^{-2.5}M$ 5-fluorouracil. The mutants exhibited restriction of growth in vitro at 37° or 38°C, and a similar restriction of growth in the hamster's lungs was observed in vivo. Infection of hamsters with either mutant induced significant resistance to subsequent challenge with parental virus (*64*). In addition, infection of mice with the more restricted mutant induced resistance to pulmonary consolidation produced by a mouse pathogenic influenza A_2 virus.

Mycoplasma pneumoniae. Steinberg detected 14 ts mutants of *M. pneumoniae* among survivors following exposure of organisms to NTG (25 to 100 micrograms per milliliter) at *p*H 7.2 (*65*). The mutants grew normally at 32°C, but exhibited a 10^{-2} to 10^{-5}, or greater, depression of colony formation on agar medium at 38°C. These mutants, although exhibiting a variable degree of "leakiness" at 37°C, were stable when grown in broth medium. Seven of the mutants were passaged five times in broth without the emergence of wild type revertants. Six of these mutants were identical to the parent strain in all properties tested except temperature sensitivity; the seventh mutant exhibited three defects in addition to temperature sensitivity, suggesting that NTG had affected more than one cistron.

Preliminary studies in hamsters with seven of the mutants have shown that these organisms grow less well in the lungs than the parent (wild type) strain does (*66*). Significantly, none of the hamsters infected with a ts mutant developed lung lesions, whereas 64 percent of the hamsters inoculated with the parent strain exhibited pulmonary pathology. Finally, infection with any of the seven avirulent ts mutants induced definite resistance to subsequent challenge with the virulent parent strain (*66*).

The experience to date with ts mutants of RS virus, influenza A_2 virus, and *Mycoplasma pneumoniae* is sufficiently encouraging to justify continued evaluation of these organisms as poten-

tial candidates for use in live vaccines. The ultimate success of the ts mutant approach to respiratory disease immunoprophylaxis will depend upon in vivo stability of the mutants and retention of their capacity to infect the upper respiratory tract of man and to grow to a level which provides adequate stimulation of the local respiratory tract defense mechanisms.

Outlook

Despite the difficulties which exist, the outlook for eventual control of acute respiratory tract disease is encouraging. Most respiratory illnesses can now be explained as being caused by viruses or mycoplasma which we are able to cultivate in the laboratory. The biology and ecology of these pathogens is reasonably well understood, and within the past few years we have gained considerable insight into the immunologic determinants of resistance to infection and respiratory illness. At the same time, awareness of the potential hazards of vaccines has reached a remarkable level of sophistication. Finally, the newer techniques of microbial genetics have been applied to the production and selection of attenuated mutants, some of which show promise as potential live vaccine strains. Thus, it appears that most of the ingredients for successful immunoprophylaxis are now at hand. The next few years cannot help but be both exciting and productive of effective respiratory tract vaccines.

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