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ORAL REHYDRATION THERAPY - RECENT ADVANCES*

CONTENTS

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1. INTRODUCTION

One of the most important advances in the field of diarrhoeal diseases research has been the discovery that dehydration in cases of acute diarrhoea of any etiology and in all age groups can be treated orally. The solution used (ORS) is prepared by adding to drinking water appropriate amounts of glucose, sodium chloride, sodium bicarbonate, and potassium chloride to give the optimum concentrations for their intestinal absorption. Even in the presence of copious diarrhoea, this solution is absorbed in the small intestine, thus replacing the acute diarrhoeal losses. Numerous studies in adults, children, infants, and neonates have established the safety and effectiveness of such oral treatment, which is therefore a simple and important public health tool.

The article below reviews the development of oral rehydration therapy during the last decade - its scientific basis and use in the treatment of diarrhoea in infants, the nutritional benefit, its application in treatment centres and field settings, and its role in national diarrhoeal diseases control activities in the context of primary health care.

2. THE SCIENTIFIC BASIS - CARRIER-MEDIATED INTESTINAL TRANSPORT

While the concept of an oral rehydration solution containing common salt and sugar is not new, the scientific basis for it was not known until the 1960's when in vitro and in vivo studies showed that glucose could mediate sodium transport across the small intestinal mucosa.[1-10] Evidence that glucose absorption and glucose-mediated sodium, chloride, and water absorption remained largely intact during diarrhoea was first produced in cholera patients in 1964.[11] In 1968, further studies in cholera patients showed that fluid and electrolyte losses due to watery diarrhoea could be adequately relaced by oral electrolyte solutions of optimum composition.[12-14]

For the optimum absorption of orally administered electrolytes and water, it has been demonstrated that certain physiological criteria have to be met:

(a) The solution (including the osmolarity contributed by glucose) should be approximately isosmotic with plasma;[10,15,16]

(b) The concentration of the carrier, i.e., glucose, should be 2-3% in order to achieve maximum sodium and water absorption; a higher concentration can induce osmotic diarrhoea[16] and a lower concentration can result in suboptimal absorption of electrolytes and water;[7-10,12-14]

(c) The nearer the sodium concentration in the solution is to its concentration in plasma, the higher is its net absorption;[10,12-14,17] a sodium concentration of 90 mmol/l in the solution induces an optimum ratio of sodium to water absorption for correcting the sodium and water deficit in diarrhoeal dehydration;[18-20]

(d) Potassium losses from acute diarrhoea, can be particularly harmful in infants, especially those that are undernourished; potassium absorption takes place passively depending on the concentration gradient, i.e., a potassium concentration higher than that in plasma will induce absorption.[21] The potassium concentration in ORS (20 mmol/l) is well tolerated and adequate for replacement therapy;

(e) Bicarbonate, which is needed to correct acidosis, is actively absorbed independently of glucose;[22,23] base deficit acidosis produced by diarrhoea of any cause and in any age group can be corrected using oral fluids with bicarbonate concentrations of 30-48 mmol/l.[12-14,20,24-30]

Composition of the oral rehydration solution recommended by WHO (ORS)

Ingredients	Grams/litre water
Sodium chloride	3.5
Sodium bicarbonate	2.5
Potassium chloride	1.5
Glucose	20.0
Composition	**mmol/litre of water**
Sodium	90
Potassium	20
Chloride	80
Bicarbonate	30
Glucose	111

Some paediatricians have expressed concern about the sodium concentration (90 mmol/l) in ORS and therefore about its use in infants. However, the generally recognized advantage of this concentration is that it is suitable for the treatment of older children and adults, in whom diarrhoea (e.g., in cholera and enterotoxigenic Escherichia coli diarrhoea) is often secretory with a stool sodium concentration of up to 130 mmol/l,[20] as well as infants and children with non-cholera diarrhoea whose stool sodium concentrations are usually lower,[31,32] but whose accumulated electrolyte losses[18,31] are well corrected by a solution having a sodium concentration of 90 mmol/l. The treatment of diarrhoea in infants is considered in detail below.

Thus, the WHO-recommended ORS formulation satisfies the physiological criteria for intestinal absorption of water and electrolytes, thereby meeting the need for optimum correction of the fluid and electrolyte deficit which occurs in infants, older children, and adults during dehydration due to diarrhoea (including cholera).

Because of the high cost or non-availability of glucose in some countries (though in others glucose is cheaper) and the desire to produce packages of the oral rehydration glucose-electrolyte salts locally, studies have been undertaken to determine whether sucrose could be substituted for glucose in the formulation. Controlled trials in a number of countries have shown that, in cholera and non-cholera diarrhoeas in all age groups, sucrose can be an adequate substitute for glucose, although its use is associated with slighly lower success rates, especially in patients with profuse diarrhoea[26,33-38] and (in one study) with more vomiting, which was thought to be related to its sweeter taste and thus faster rate of ingestion.[38] In all these studies, in order to obtain the same osmolarity as glucose, the sucrose concentration in grams per litre was twice that of glucose. One can conclude from the available information that, from a physiological standpoint, glucose and sucrose are both effective, but when both are readily available, glucose is preferred.

3. ORAL REHYDRATION THERAPY IN INFANTILE DIARRHOEA

Infants (defined here as children under 2 years of age) with diarrhoea require special attention during:

(a) the rehydration phase, i.e., the replacement of the water and electrolyte deficit present at the time of starting therapy; and

(b) the maintenance phase, i.e., the replacement of continuing losses (due to diarrhoea and vomiting) and the provision of additional plain water requirements. The plain water requirement is due to the relatively larger surface area and higher metabolic rate per unit of body mass in infants, who therefore require about two and a half times more water per kilogram of body weight than adults.[32]

As mentioned above, the optimum electrolyte concentration in ORS facilitates the process of rehydration in these infants. During maintenance also, ORS should be administered to replace the ongoing diarrhoeal losses; additional free water requirements should be met, preferably by encouraging breastfeeding (breast milk has a sodium concentration of about 7 mmol/l)[39] or by giving plain water or dilute milk feeds. Thus, the strategy for the administration of ORS to infants rigorously meets the well-known principles of fluid therapy based on meticulous balance studies[18,31] and the known physiological requirements of infants.[32]

A number of studies have recently confirmed the safe use of oral rehdyration therapy in the treatment of infantile non-cholera diarrhoeas. These include studies conducted in Bangladesh,[29,34,37,38,40] Brazil,[49] Congo,[42] Costa Rica,[28,46,47] India,[26,27,43-45] Jamaica,[48] Nigeria,[41] and the United States of America.[24,25] Several of these studies (in which the cause of the diarhoea was determined) showed that oral rehydration therapy using ORS was extremely effective in treating diarrhoea caused by rotavirus, an invasive pathogen that is known to cause considerable disruption of the intestinal mucosa.[50] In many of these studies, as well as in studies in older children and adults, vomiting occurred not infrequently during the course of therapy but it was not an obstacle to the successful use of oral rehydration therapy.

A recent study in Costa Rica has clearly demonstrated for the first time the safety and efficacy of ORS in the treatment of acute diarrhoea in neonates: 39 of 40 neonates with moderate to severe dehydration were successfully rehydrated and maintained in water and electrolyte balance using ORS.[30]

Included in some of the above-mentioned studies were infants and children with moderate and severe malnutrition (also patients with classical marasmus and kwashiorkor); it was demonstrated that ORS therapy was as effective and safe in these as in well-nourished children.[26,27,38,40]

4. NUTRITIONAL BENEFITS OF ORAL REHYDRATION

Hospital studies initially suggested that children whose dehydration, acidosis, and potassium deletion were soon corrected with oral rehydration therapy resumed feeding earlier and regained more rapidly the weight lost during the diarrhoea.[51] A subsequent field trial conducted in the Philippines showed

that infants who received oral rehydration therapy together with proper dietary management (using the available foods) during and after the diarrhoea had a better appetite and gained significantly more weight than controls over a 7-month period of observation.[52] Similar studies subsequently carried out in Egypt,[53] Iran,[54] Liberia[55] and Turkey[56] showed a similar trend in weight gain in children who received oral rehydration therapy and dietary management during episodes of acute diarrhoea. Prepackaged ORS was used in all these studies. The precise mechanism of the weight gain is not clear, although correction of acidosis and replacement of potassium losses probably played a role in hastening the improvement of appetite.

All these studies indicate that oral rehydration therapy may have an important nutritional benefit, and emphasize the value of a complete rehydration formula and proper dietary education of mothers.

5. EXPERIENCE WITH ORAL THERAPY IN TREATMENT CENTRES

Since the early studies in Bangladesh and India, oral rehydration has gained worldwide acceptance and use in treatment centres. One of the earliest and most dramatic demonstrations of its efficacy and benefit was its use, under the most difficult field conditions, in the maintenance of hydration in severely dehydrated Bangladesh refugees during the 1971 civil upheaval.[57] Many countries in Africa and Asia had their initial experience with oral rehydration therapy in cholera outbreaks (during the present pandemic) and obtained remarkable successes, e.g., during the cholera outbreak in the Maldives in 1978.[58]

Studies in some countries have shown that the use of oral rehydration therapy can result in a great reduction in the amount of intravenous fluid used in the treatment centres. For example, two hospitals in India reported savings of 70% of the expenses previously incurred for intravenous fluid after appropriate use of oral fluid.[45,59] There are also some data indicating that reduction in the use of intravenous therapy can be accompanied by a marked decrease in nosocomial infections and hospital mortality in diarrhoea cases.[60,61] There is no doubt that intravenous fluid will continue to be needed in treatment centres for the rehydration of diarrhoea cases with severe dehydration or other complications (e.g., persistent vomiting, poor mental state, concurrent infections), but these cases constitute no more than 5-10% of all cases seen at such centres.

6. DELIVERY OF ORAL REHYDRATION AT THE PERIPHERY

Increasing attention is now being focused on extending the delivery of oral therapy, as a primary health care activity, to the village and household levels, where an even greater impact on reducing the mortality in children under five years of age can be expected. Therapy at these levels, if applied properly and early in the course of the illness, can prevent the development of severe dehydration and thus reduce the need for intravenous therapy and hospitalization.

Field evaluations of the effectiveness of early oral rehydration therapy are being undertaken. In a limited study carried out in Bangladesh, home delivery of oral rehydration solution by trained village health workers resulted in a reduction of 29%, over a 4-month period, in the number of cases seeking therapy at a rural treatment centre.[62] A more recent study in Bangladesh, carried out over a longer period, showed a significant reduction in case-fatality rates when ORS packets were made easily available through trained village-based volunteer workers and after a short promotional campaign had been conducted to inform the village population.[40] Other field evaluations of oral rehydration therapy are now being carried out or are planned as an integrated activity in populations served by multi-purpose village health workers in a number of countries, including Bangladesh, Costa Rica, Egypt, India, and Nepal.

Other studies conducted in urban environments in Costa Rica[47] and Jamaica[48] have shown that oral rehydration can be carried out successfully in the home. In these studies, nurses' aides instructed and enlisted the help of mothers in the oral rehydration of their acutely ill infants during visits to a hospital emergency room. Mothers were then taught how to mix and administer ORS at home and how to assess dehydration from skin elasticity. In both studies, 85-90% of the infants were satisfactorily managed at home by their mothers, and only half the infants who were brought back to the emergency room during their illness required intravenous fluids.

Some public health workers, realizing the benefits of early administration of oral rehydration during illness and faced with the difficulty of making it available where it is needed most, have attempted to simplify the composition of the mixture and its means of delivery in field settings. For example, the use of ordinary teaspoons has been advocated in the preparation of a simplified rehydration solution in the home using domestic salt and sugar. However, because of the variation in spoon sizes as well as the quality of the ingredients and the capabilities of mothers, the use of teaspoons has been shown to result in dangerously high concentrations as well as low, ineffective levels of sodium in the fluids so prepared.[63,64] Estimation of the amount of salt (with a 2-finger-and-thumb pinch) and of sugar (by a 4-finger scoop), as is commonly practised for cooking purposes, has also been advocated but has also been found to give large variations in the concentration of the ingredients.[63,65] The size of the grains, the amount of dampness present, individual dexterity, and different cultural use of the fingers were some of the important factors contributing to the variability.[66] As a possible means of increasing the accuracy of measurements in the home, a number of special plastic two-ended measuring spoons have been devised.[67,68] These spoons are undoubtedly more accurate, although a recent study in Papua New Guinea showed that 17% of well-trained mothers prepared solutions with an unacceptably high sodium concentration which was due in large part to the small size of the vessels used for measuring the quantity of water recommended.[69] Another potential problem is the non-availability of ingredients. Initial data from a study of household-prepared rehydration solutions in Nepal showed a shortage of crystalline sugar in some parts of the country.[70] All these studies illustrate that there can be contraints in the preparation of oral rehydration solutions from household ingredients which must be carefully considered and evaluated.

In a large field programme in Punjab, India, the use of a salt-sugar solution by village health workers and mothers for the early treatment of diarrhoea led to a reported 50% decrease in diarrhoea mortality.[71] Additional studies on the safety and efficacy of these simplified procedures in the home are planned or are under way in Bangladesh, Egypt, Honduras, Indonesia, and Nepal, and are likely to provide much-needed information.

It is evident that the complete formulation should always be used for the treatment of cases with significant dehydration. This has recently been demonstrated in studies in Bangladesh[72] and Honduras[73] in which both adults and children with moderate dehydration who were treated in a hospital setting with an oral rehydration solution made from common salt and unrefined household sugar had more prolonged acidosis or severe hypokalaemia than those treated with ORS. There is also the theoretical concern that these salt-sugar solutions may not provide the nutritional benefit seen with the complete formula, because they lack bicarbonate and potassium. It is also believed by many that the packets containing the pre-packaged ingredients serve as a very successful carrier of messages on feeding and hygienic practices.

7. CONCLUSIONS

Research on intestinal absorption and the transport mechanisms involved has led to the development of an optimum oral rehydration solution, which is well absorbed by subjects of all age groups during diarrhoea. Balance studies using different formulas and therapeutic regimens have helped to establish practical therapeutic methods, and the adoption of these methods in hospitals has led to a marked reduction in intravenous fluid use and costs. Initial studies indicate that easy availability of oral rehydration therapy in rural areas can dramatically reduce the case fatality rate (due to acute diarrhoeas) and the number of visits to treatment centres; more field evaluations are needed to confirm and expand these observations.

It is clear that ORS is an eminently suitable tool for application at the primary health care level, and an excellent entry point for health education activities to promote other preventive measures - not only those that are important for the prevention of diarrhoea (e.g., breastfeeding, appropriate weaning practices, and good personal hygiene in the family), but also other critical health interventions (e.g., immunizations, family planning, etc.). Experience has shown that during the illness of her child with diarrhoea, the mother is likely to be very receptive to such advice.

Development of national diarrhoeal disease control programmes to ensure the continued delivery and production of ORS and the training and education of health workers and families is now needed so that the mortality and other sequelae of acute diarrhoeal diseases can be prevented.

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Intestinal immunity and vaccine development: a WHO Memorandum *

As part of the research component of the WHO Diarrhoeal Diseases Control Programme, a Scientific Working Group met in August 1978 to review recent advances in knowledge of intestinal immunity, the application of genetic techniques in enteric vaccine development, the status of currently available immunizing agents against cholera, typhoid fever, and Shigella *dysentery, and the prospects for the development of new or improved vaccines against the well known and newly recognized agents such as rotavirus and enterotoxigenic* Escherichia coli. *In each of these areas, the Group made specific recommendations for further research that should be considered for support under the WHO research programme in the field of diarrhoeal diseases.*

The ultimate control of the infectious diarrhoeas and other enteric infections can best be achieved through improvement of water supplies, sanitation, and education. Because of limitations of resources and other competing priorities, this goal will not be realized in many parts of the world in the near future.

A complementary approach to prevention is the development of vaccines that can confer long-lasting protection. An example of the beneficial impact of an enteric vaccine was the development of both living and inactivated vaccines against poliomyelitis which have markedly reduced the worldwide incidence of this disease.

The development of effective vaccines against the major enteric pathogens will require better understanding of immune mechanisms in the gastrointestinal tract. Much progress has recently been made in our understanding of gut-associated immunity, but more basic information in this area is needed.

In recent years, important advances have also been made in the delineation of the mechanisms of pathogenesis of some of the infectious diarrhoeas, but much remains to be explained.

This Memorandum discusses recent advances in knowledge of gut-associated immunity, the application of new genetic knowledge to enteric vaccine development, the status of currently available immunizing agents and the prospects for their improvement and for development of new ones.

* This Memorandum was drafted by the signatories listed on page 734 on the occasion of a meeting of the Scientific Working Group on Immunity and Vaccine Development, Geneva, 14-16 August 1978. Requests for reprints should be addressed to Diarrhoeal Diseases Control Programme, World Health Organization, 1211 Geneva 27, Switzerland. A French translation will appear in a future edition of the *Bulletin*.

INTESTINAL IMMUNITY

The intestine is a major immunological organ, containing in its lamina propria as many lymphoid cells as the spleen. These cells include T cells (thymus-derived), B cells (bone marrow-derived), null cells (small lymphocytes lacking the features of B or T cells), and plasma cells. The latter are mostly of the IgA class and are the source of antibody secreted to the mucosal surface. The numerous small lymphocytes participate in control of antibody production and also undoubtedly mediate important cellular immune responses which are still poorly understood. It appears that secreted antibody is the major protective mechanism against enteric viruses and non-invasive bacteria or their toxic products. Antibodies, secreted and systemic, are probably also protective against invasive bacteria, but cell-mediated immune mechanisms are also undoubtedly important. This review emphasizes the immune response leading to production of secretory antibody, primarily because this part of the intestinal immune response is best understood. Most of the concepts included are derived from animal studies; these probably apply generally to humans, but species differences may be encountered.

INTESTINAL ANTIBODIES

Antibodies that protect the mucosa of the bowel against non-invasive organisms or their products can be derived from two sources: serum and plasma cells in the intestinal lamina propria. The mechanism for deriving antibody from serum appears inefficient because protective amounts of antibody are present only when serum antibody titres are high, and these can rarely be sustained. Serum antibody that makes its way into the lumen of the gut is predominantly IgG. Antibodies produced locally by plasma cells in the lamina propria, however, are usually IgA; they are selectively secreted on the mucosal surface by the crypt epithelium. Since secretory IgA resists proteolysis by intestinal enzymes, it appears better designed for protection of the mucosal surface than IgG.

To a large extent the mucosal immune system functions independently of systemic immune mechanisms. An immune response can be elicited in either system without necessarily producing an immune response in the other. To produce a local immune response, it appears that antigen must reach the lymphoid tissue of the gut, in particular the Peyer's patches. This contact sometimes occurs with parenteral injected antigens, especially when they are given in large doses. Usually, however, vigorous local responses are not induced unless the mucosal surface is directly exposed to antigen.

Peyer's patches are discrete follicular collections of lymphoid cells located in the submucosa of the intestine; they contain T cells, B cells, and macrophages. Peyer's patches are found in the small bowel and the appendix and contain the precursor cells for an IgA response. The mucosal epithelium overlying Peyer's patches is modified, forming only rudimentary villi and displaying increased pinocytotic activity. Although small quantities of macromolecules, including proteins, are absorbed intact by the villi, the mucosa overlying Peyer's patches seems designed for enhanced "sampling" of antigens from the gut lumen and "presentation" of these antigens to lymphoid tissues in the patches. Viruses that replicate in the mucosa are usually highly immunogenic. On the other hand, non-living antigens vary considerably in their efficiency at inducing a local immune response. For example, many milligrams or grams of proteins such as bovine serum albumin or horse spleen ferritin may be needed to produce a response, whereas 100 ng of cholera toxin elicits a primary response in rat gut. These differences, although not well understood, may be due to specific properties of cholera toxin which make it a particularly effective mucosal antigen. These include membrane binding, which may enhance trapping of adsorbed antigen by Peyer's patch lymphocytes, and adenylate cyclase activation, which may enhance the immune response by a direct effect on lymphocyte function. If other antigens could be selected for, or designed to have, these features they might be particularly effective as mucosal immunogens.

KINETICS OF THE IGA RESPONSE AND PRODUCTION OF IGA ANTIBODY

The origin and migration of enteric IgA plasma cells, or their precursors, are shown in Fig. 1. This illustration summarizes work from several laboratories and, although protein antigens have been studied in greatest detail, it probably applies to responses to all types of antigen.

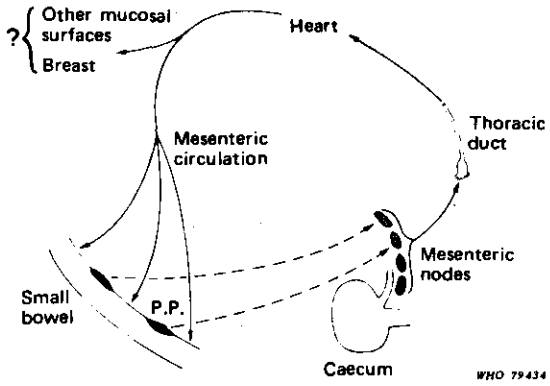

Fig. 1. Circulation of enteric immunocytes. Immunoblasts originate in Peyer's patches (P.P.) or mesentric nodes, migrate through the thoracic duct, and enter the systemic circulation. Homing is predominantly to the intestine but possibly also to other mucosal surfaces or to the breast. In the lamina propria immunoblasts appear as plasma cells. Reproduced from PIERCE, N. F. Intestinal antibodies. *Journal of infections diseases,* **137**, 661-662 (1978), by kind permission of the author and the publisher, the University of Chicago.

In unprimed animals, effective encounter of antigen with Peyer's patch lymphoid tissue causes expansion of a clone of B cells committed to production of specific antibody of the IgA class. A few of these cells are transformed to plasmablasts and leave the patch to enter the efferent lymphatics; most, however, remain in the patch as antigen-sensitized B cells. Sensitization of T cells probably also occurs but has not been studied in detail. When a second antigen exposure occurs these sensitized cells rapidly undergo blast transformation. This is followed by cellular division and subsequent migration of IgA immunoblasts, first to mesenteric lymph nodes and then through the thoracic duct to the systemic circulation. Hence, IgA cells generated from a small area of the gut can disseminate throughout the host. It is likely that some lodge in distant IgA-secreting sites such as the lamina propria of the respiratory tract or the ductal epithelium of the breast. The vast majority, however, return to the lamina propria of the bowel, where they rapidly assume the morphology of plasma cells and have an average life span of about five days. The presence in the mucosa of antigen against which their antibody is directed is a major determinant of the selective "homing" of these cells to the bowel. In contrast to the primary response, this secondary type is large and rapid, appearing within 48–60 hours, reaching a peak at 4–5 days, and declining rapidly thereafter. When the immune response is induced by prolonged exposure, as occurs in viral or bacterial infection of the gut, the primary and secondary features merge to form a continuum of events until the infection is ended.

IgA-producing cells in the lamina propria secrete a 9S IgA dimer that enters epithelial cells in the crypt region. The IgA is then combined with "secretory piece" produced by these epithelial cells, and the immunoglobulin is secreted as 11S secretory IgA on the mucosal surface. Secretory IgA antibodies provide antibacterial protection by two general mechanisms: (1) direct action on bacteria, which results in immobilization, agglutination, or prevention of their adherence to the mucosa; and (2) combination with bacterial products, such as toxins or enzymes, which causes inactivation and assists in their destruction by proteolytic enzymes. There is no evidence that secretory IgA plays a significant bactericidal role. Secretory IgA exerts antiviral activity by combining with virus and preventing its uptake by host cells.

DURATION OF MUCOSAL PROTECTION

There are two ways by which prolonged protection mediated by local antibody might occur at the mucosal surface. First, it is possible that specific IgA plasma cells produce small but protective amounts of secretory antibody which continues to be generated long after antigen exposure ends. Second, it is possible that prolonged protection is dependent on rapidly activated immunologic memory in the mucosal IgA system. The demonstration of primary- and secondary-type responses in this system is evidence for a kind of local immunologic memory. Such a response could protect against infections with an incubation period of 3–5 days or longer, and might also accelerate the termination of established infections. It is less certain that the secondary-type response could be sufficiently rapid to prevent diseases with shorter incubation periods, a feature of many viral and bacterial enteric infections. The maximum duration of local memory with undiminished secondary responses is uncertain but it can last at least 4–8 months. The magnitude of the secondary response may be diminished and memory may be much shorter, however, owing to the emergence of antigen-specific suppression of the IgA response. The mechanism of this suppression is uncertain but it usually increases in strength with time after immunization, often taking 4–8 weeks to reach a maximum. It may persist for many months. In rats and rabbits it is often seen after parenteral

immunization and may also follow intestinal immunization; in contrast, it has not been observed in parenterally immunized dogs. Neither memory nor suppression have been studied in the mucosal IgA system of humans.

TECHNIQUES FOR MEASURING THE MUCOSAL IMMUNE RESPONSE IN HUMANS

Techniques for measuring the mucosal immune response must be based on an understanding of the characteristics of this system, especially the distribution and class of antibody produced. Traditional methods for studying the systemic immune response depend heavily on detection of antibody in serum; except as noted below, these are of little or no value in measuring the mucosal immune response. Several methods that might be used are described below. Also, it should be noted that a mucosal immune response may or may not be associated with protection, and protection may be present when no immune response can be detected. The correlation between protection and the measurement of a specific local response may be demonstrated only by challenge studies or appropriate prospective field studies.

Direct measurements

Specific secretory antibody may be detected in intestinal fluid obtained by intubation. The major difficulties are (1) obtaining the specimen and (2) the degree of dilution of secreted IgA by intestinal juices. Secreted antibody may also be partially degraded by intestinal enzymes. In some assays unmodified intestinal fluid may be used but in others extensive dialysis, concentration, or immunoglobulin separation is required before suitable results can be obtained. In general, the assay method should be specific for the IgA class of antibody to exclude detection of serum-derived antibody. The enzyme-linked immunosorbent assay system (ELISA) has proved to be very useful for this purpose. Assays that detect the function of antibodies, for example, neutralization of viruses or toxins, are also important but are usually not specific for antibody class.

Specific antibody-containing plasma cells have been identified and counted in lamina propria biopsies of animals using an antigen-specific fluorescent antibody technique. This might be used in humans but the difficulties of intestinal biopsy and the small sample size severely limit its application.

Indirect measurements

Measurement of specific IgA antibody in other external secretions, especially saliva, colostrum, and milk, may prove useful. This approach is based on evidence that the intestinal IgA response is disseminated, in part, to these sites. This approach has the advantage of easier sample collection. It is still not certain, however, that the intestinal IgA response is reliably or quantitatively reflected in these secretions. This may be clarified by further studies.

Measurement of specific IgA antibody in serum is also of value because some intestinal IgA is not secreted, but enters the blood stream. This approach requires an assay that is highly specific for IgA, since specific antibody of other classes and derived from non-mucosal sites is likely to be present. Additionally, it is not certain that specific serum IgA antibody will quantitatively reflect mucosal production since 7S monomeric IgA is also produced in the spleen.

CELL-MEDIATED IMMUNITY AND NON-IMMUNOLOGICAL FACTORS

It should be emphasized that secretory antibody is only one component of a complex enteric defence system. Cell-mediated immunological defence mechanisms are poorly understood but undoubtedly respond to and protect against invasive bacteria and possibly viruses. Extensive study of this portion of the mucosal immune system is required. Finally, non-immunological factors such as gastric acid, proteolytic enzymes, peristalsis, and mucus secretion are also important in preventing bacterial colonization or inactivating toxic bacterial products.

APPLICATION OF GENETIC KNOWLEDGE IN ENTERIC VACCINE DEVELOPMENT

THE GENETIC ANALYSIS OF ENTERIC PATHOGENS

Microbial genetics focuses upon the identification of specific virulence traits through examination of mutants deficient in one or more genes contributing to pathogenicity. These determinants are then characterized by genetic crosses. Since the early 1960s, genetic procedures, first carried out in

Escherichia coli K-12, have been available for the study of enteric pathogens. Hence, inter-species and inter-genus recombinants between *E. coli, Shigella,* and *Salmonella* may be produced by transduction (bacteriophage-mediated transfer of genetic material) and conjugation (genetic transfer through pili by cell-to-cell contact). More recently, transformation (genetic transfer mediated by the uptake of free DNA molecules) has been used for the analysis of *E. coli* K-12. This procedure has not been widely applied to other Enterobacteriaceae nor to *Vibrio* spp.

The transmission of chromosomal genes between different organisms by transformation is often limited in scope because of differences in DNA homology or in specific enzymes that degrade (restriction) or modify (modification) incoming foreign DNA. Yet the application of transfer procedures has been useful in several instances. For example, conjugation and transduction studies between *E. coli* and *Shigella flexneri* have led to the identification of specific genetic determinants which control colonization of the bowel, invasion of epithelial cells, and intracellular multiplication. Several *E. coli–Shigella* recombinant strains were developed as potential vaccine strains by these genetic procedures.

In a few studies attempts have been made to identify the genetic determinants of pathogenesis of *Salmonella* infection in mice and considerable progress has been made in understanding the genetic basis of antigen biosynthesis. A genetic transfer system has also been available in *Vibrio cholerae* since the early 1960s. Nevertheless, there is very little information on the genes in *V. cholerae* that control toxin biosynthesis and govern the ability of vibrios to colonize the gut. Recently, as a result of the availability of new and better donor and recipient strains as well as the application of some of the more recent advances in microbial genetic technology, research on the genetic analysis of *V. cholerae* has been increasing.

Suitable genetic systems are now available for the study of the pathogenesis of *E. coli, Salmonella, Shigella,* and *V. cholerae* diarrhoeas. These systems have not been vigorously applied until recently owing to the lack of relatively simple tests to detect the products of virulence genes among the large numbers of recombinants issuing from genetic crosses. It is also true that, with a few notable exceptions, those with the greatest genetic expertise have not been interested in the genetics of the determinants of pathogenesis.

By the same token, those most interested in developing vaccine strains have often been unaware of available genetic methodology.

PLASMID-MEDIATED DETERMINANTS OF VIRULENCE

Plasmids, extrachromosomal elements that can be transferred from one bacterium to another, have been studied intensively over the past 20 years. Most of the studies have been concerned with plasmids that determine antibiotic resistance. More recently it has been recognized that *E. coli* possesses plasmid-mediated determinants of virulence which include enterotoxins (*Ent*$^+$) and colonization factors. Both determinants are required for full pathogenicity. The chromosomal determinants that act in association with *Ent*$^+$ and colonization antigens remain largely unknown. Such contributing factors may be of considerable importance in developing appropriate vaccine strains.

The prospect for genetic manipulation of *Ent* and colonizing plasmids is excellent. For example, it is possible to transpose antibiotic resistance genes onto these plasmids. This serves not only to "mark" the plasmid with a readily definable marker but also allows for the isolation of non-toxinogenic (*tox*$^-$) derivatives which may be deficient in the biosynthesis of one or more structural or regulatory genes. It should not be difficult to isolate a mutant that produces an immunogenic but non-toxic product that might be useful in vaccine development.

There is very little published information on the possible contribution of plasmids to the pathogenesis of salmonellosis, shigellosis, or cholera.

TRANSPOSITION

One of the most intriguing recent findings in microbial genetics has been the identification of a class of base sequences that can be excised from one portion of the DNA molecule and inserted into another DNA site within the same cell. Many R factor-mediated antibiotic resistance genes behave in this way. These resistance segments, called transposons or transposition sequences, can be excised from an R factor genome and inserted into another plasmid residing within the cell. They may also transpose into the bacterial chromosome. These transposition events occur at a rather high frequency and virtually at random.

The insertion of a transposon is mutagenic in that it disrupts the function of the gene into which it has been inserted.

The implications for the exploitation of transposition are many. For example:

(1) it can be employed for the isolation of mutants in virtually any plasmid or chromosome gene;

(2) R factors carrying transposons can be widely transferred to all Enterobacteriaceae and to *V. cholerae*;

(3) distinct transposons for antibiotic or heavy-metal resistance can be used to mark a single strain or plasmid in multiple sites;

(4) under some conditions, transposons can be forced into the chromosomes so that every resulting bacterial clone will possess a chromosomal insertion. This may have a distinct advantage over chemical mutagenesis in that there is a high probability of success of finding any particular class of mutants;

(5) it is possible to construct strains carrying identical transposons on the bacterial chromosome and on a resident plasmid. Under appropriate conditions these strains can be employed as genetic donors.

"MINICELLS"

There exists a class of mutants termed "minicells" in *E. coli, Salmonella,* and *Shigella* which may represent a promising novel approach to vaccine development. These are small, non-viable cells, easily obtainable in large quantities, which are the products of aberrant cell division. Chromosomal DNA does not segregate into "minicells" although they are normal in most other respects, including cell wall structure and cellular appendages.

RECOMBINANT DNA

Recent advances in DNA biochemistry and the development of recombinant DNA technology have significantly increased prospects for the isolation and genetic manipulation of virulence genes. Recombinant DNA molecules may be formed *in vitro* by the enzymatic joining of DNA fragments from virtually any source to a carrier replicon such as a plasmid or bacteriophage. Once such molecules are formed *in vitro,* the technology exists for introducing the recombinant DNA molecules (also called chimeras) into a suitable bacterial host where the chimeric molecule will replicate.

Recombinant DNA methods have been applied successfully to the isolation of the *E. coli* ST, LT, K88, and K99 genes. For example, the structural gene for ST biosynthesis has been sequenced and the amino acid sequence of ST deduced. In addition, the nature of the ST gene sequence has led to the discovery that the structural gene for ST resides upon a transposon. These data would suggest that the ST genes in many different species have a common origin. Moreover, as noted elsewhere in this Memorandum, a simple *in vitro* test for the detection of ST is greatly needed. Since one may rearrange the position of the ST gene by judicious use of joining enzymes, it may prove possible to fuse the normally non-immunogenic ST polypeptide to an immunogenic protein. This could be a useful means of developing a serological test for detecting ST and possibly an immunizing agent against the ST product.

These methods may be applied to any gene of interest. While the recombinant DNA methods have their limitations, they are clearly designed to focus upon only a few genes and their products. The methodology has potential implications for the immunoprophylaxis of enteric disease since it permits one to design the modification of specific genetic sequences. Recombinant DNA technology has already been applied to the *in vitro* synthesis of several eukaryotic hormone genes and perhaps will soon be applied to the synthesis of prokaryotic genes which may produce appropriate immunogens.

THE ISOLATION OF SPECIFIC MUTANT STRAINS FOR USE AS VACCINES

In principle, live oral vaccine must (*a*) be non-pathogenic for man, (*b*) be stable, (*c*) provide protection, and (*d*) carry one or more appropriate markers for its identification in the host and the environment. For non-invasive enteric pathogens such as *V. cholerae* and enterotoxigenic *E. coli,* it should retain its ability to colonize the gut epithelial surface, but for organisms that multiply intracellularly like *Shigella* and *Salmonella typhi,* this capacity to enter and multiply within cells may not be separable from their ability to cause disease. A good deal of effort has been directed toward isolating specific classes of mutants which might possess these qualities. Most of this work has involved *Shigella* and *Salmonella* species and, in the main, the mutants studied have been conditionally lethal, i.e., mutants have been isolated that may be readily grown in the laboratory under selective conditions, but rapidly lose their capacity for multiplication within the host animal. This feature limits the antigenic mass available for stimulation of the immune systems. The best known of such mutant classes have been streptomycin-dependent derivatives of *E. coli, Shigella, Salmonella,* and *V. cholerae.* The precise genetic lesions in these streptomycin-dependent mutants are un-

known, although they could presumably be analysed by conventional genetic procedures.

It would be desirable to examine candidate vaccine strains not only for their capacity to revert, but also for their precise genetic defect. The genetic lesion in such candidates should preferably result from a deletion of genetic material or from a series of point mutations within the same gene. Moreover, it is only by precise genetic analysis that one can accurately locate a specific gene.

STATUS OF CURRENTLY AVAILABLE IMMUNIZING AGENTS AND PROSPECTS FOR DEVELOPMENT

IMMUNOGENS FOR CHOLERA AND ENTEROTOXIGENIC *ESCHERICHIA COLI*

Seroepidemiology of cholera

In areas of high endemicity cholera is mainly a childhood disease. Studies in Bangladesh have compared seroepidemiological data and disease occurrence. Incidence was low below 2 years of age, maximal at 2–5 years, and fell steadily with increasing age. The proportion of healthy persons possessing vibriocidal antibody rose rapidly during childhood so that by the age of 12 years about 80% showed evidence of prior infection; this high level was maintained throughout the rest of life. Serum antitoxin, however, reached a peak in early childhood and steadily decreased thereafter. This pattern appears paradoxical since vibriocidal data indicate that repeated infection occurs throughout life. A possible explanation may be that there is unequal exposure to toxin and somatic antigens (probably lipopolysaccharide). This could result if local antibacterial immunity restricts bacterial growth, resulting in little or no toxin production. Alternatively, local antitoxin may have neutralized locally secreted toxin and prevented its absorption. It should be borne in mind that only vibriocidal and antitoxic antibodies in serum were measured. Therefore the seroepidemiology of cholera is thus far incompletely defined. An understanding of the distribution of local and systemic antibodies against other virulence factors might provide a much clearer picture of the host–parasite interaction.

Whole-cell vaccines against cholera

The protection afforded by currently available vaccines against cholera is incomplete and of short duration. The only vaccines that are available for general use are killed, whole-cell vaccines for parenteral injection. These vaccines usually contain about 4000 million killed vibrios of both Inaba and Ogawa serotypes per dose. As with all whole-cell vaccines prepared from Gram-negative bacilli, there is a fairly high incidence of local redness, tenderness, and pain following injection, as well as a low incidence of mild systemic reactions consisting of fever and malaise. The number of severe reactions is negligible. Virtually all recipients develop circulating vibriocidal antibodies (complement-dependent) and agglutinins, but no antitoxin. For vaccine manufacture, vibrios are usually grown on agar surfaces. This yields vaccines that contain virtually no intra- or extracellular enterotoxin.

Several well controlled field trials in cholera endemic areas in Bangladesh and the Philippines have shown that cholera vaccines without adjuvant afford about 50–80% protection for 3–6 months depending upon age group, quality of vaccine, and dosage schedule. Protection has been equally effective against homologous and heterologous biotypes. Protection in children below the age of 5 years may be increased by administering two doses one month apart. Recent field trials in India and Indonesia suggest that aluminium adjuvant may enhance the protection afforded by a single dose of vaccine in this particularly important under-5 age group. Nevertheless, the level of protection afforded by all types of whole-cell cholera vaccine, even in endemic areas, is of very limited public health value. None of the vaccines tested appears to have had any effect on the frequency of inapparent infections, nor have they been useful in controlling epidemics.

Experimental cholera immunogens

An Ogawa lipopolysaccharide vaccine was field-tested in Bangladesh. Subcutaneous injections conferred significant protection for one year against Inaba cholera in adults only, although the number of cases was small. In another trial in Bangladesh a whole-cell Ogawa vaccine was ineffective against Inaba disease. A lipopolysaccharide–protein complex prepared from Inaba organisms and an Inaba whole-cell vaccine afforded significant protection to adults against homologous Inaba disease for a relatively short period. Its poor performance in children

again suggested that vaccine served as a booster in adults in this endemic area and would probably not be protective in immunologically virgin populations in the doses and schedules employed. The same Inaba whole-cell vaccine also protected against Ogawa disease in the Philippines almost to the same extent as the Ogawa whole-cell vaccine, which had failed against Inaba disease in Bangladesh.

The immunogenicity and efficacy of purified cholera toxoids have been extensively studied in several animal models and in field trials. Toxins have been detoxified with both formalin and glutaraldehyde.

A field trial to test the efficacy of a parenterally injected, highly purified glutaraldehyde toxoid was carried out in Bangladesh in 1974. Although this toxoid evoked levels of circulating antitoxin at least as high as those seen in convalescence from clinical cholera, the protection afforded was very slight and transient. Significant protection was seen only in the 5–14 years' age group for about 90 days. This trial clearly demonstrated that antitoxic immunity evoked by this parenterally injected toxoid did not afford a level of protection that would be of value as a public health measure. This trial did not rule out the possibility that other forms of the toxin antigen, administered by other routes, could confer protection. Especially, it did not rule out the possibility that local secretory antitoxic antibody might play a role in resistance.

Volunteer studies of cholera and cholera immunization

Extremely valuable information concering immunity in cholera has been obtained from studies in volunteers challenged with live vibrios. The most important fact is that recovery from induced disease confers virtually 100% protection against rechallenge for at least 2–3 months. The duration of immunity beyond this period has not yet been adequately determined. This resistance is equally solid against serotypically heterologous and homologous strains. Colonization appears to be inhibited, since challenge vibrios cannot be recovered from the small bowel of resistant volunteers. While it is possible that enterotoxin is the main common antigen responsible for this protection, the apparent inhibition of colonization suggests that other non-toxin antigens are involved.

Immunization of volunteers with very large (8–24 mg), multiple, oral doses of glutaraldehyde cholera toxoid conferred no protection against live oral vibrio challenge. Very low levels of serum antitoxin were evoked in about two-thirds of the volunteers, but local antitoxin response was not determined. In contrast, in natural and induced disease, serum antitoxin responses of much greater magnitude nearly always appear, suggesting that differences in the form of the toxin antigen, the method of presentation to the mucosa, and/or the dose and schedule of delivery are responsible for the differences in antitoxin response seen between oral toxoid and infection.

Killed oral cholera vaccines have also been tested in volunteers. With the very large dosage schedule employed, there was some protection against live challenge, but it was much inferior to that conferred by prior disease.

Massive oral doses of a living hypotoxinogenic mutant of strain 569 B (M-13) conferred only 60% protection in volunteers. This relatively poor protection, as compared with that conferred by fully virulent strains, could be due to an associated defect in one of the colonization factors, or it may suggest that toxin production sufficient to evoke disease is required for optimal production of whatever protective antigen is involved.

From the findings in these volunteer studies, one can therefore say that the goal of vaccine research is clear: to develop a vaccine and means of delivery that can mimic the high order of protection provided by disease.

Diarrhoea caused by enterotoxigenic E. coli *(ETEC)*

Diarrhoeal disease induced by oral infection of volunteers with an *E. coli* strain that produced both heat-labile (LT) and heat-stable (ST) enterotoxins conferred strong protection against rechallenge with the same strain. In contrast, there was no protection against *E. coli* of another serotype producing only LT. These observations suggest that in diarrhoea caused by ETEC, immunity against non-toxin factors may play an important role in protection. As in cholera, the duration of immunity is unknown. No vaccines against ETEC diarrhoea are available for human use.

Recent epidemiological studies have clearly shown that ETEC is an important cause of "travellers' diarrhoea" in different parts of the world. Thus the development of effective prophylactic immunogens could have an important impact in reducing both endemic diarrhoeal disease incidence and disease associated with travel into new areas.

Pioneer work in the pathogenesis, genetics, and immunology of ETEC disease was done by veterinarians studying similar diarrhoeas of domestic ani-

mals. Better understanding of ETEC disease will require continuing collaboration and interchange of research experience among scientists investigating human and animal disease.

For example, immunization of dams with the colonization factor antigen K-88 conferred protection against living challenge with ETEC in the suckling piglets. This supports the limited observations in volunteers that antibodies to somatic antigens alone can afford protection against ETEC diarrhoea.

There are probably important differences in pathogenetic and immune mechanisms between diarrhoeal disease caused by the so-called enteropathogenic strains of *E. coli* (EPEC) and ETEC. In considering immunization against ETEC infections, however, it will be important to bear in mind the results of limited studies of vaccination against EPEC disease. In a study carried out in Hungary, for example, it was shown that, in newborn children, repeated oral administration of killed whole-cell vaccines prepared from the classical EPEC serotypes (055, 086, and 0111) conferred up to 75% protection against naturally occurring EPEC diarrhoea from the second to the sixth month of life. The protection during the first month was minimal.

Prospects for vaccine development

Studies in experimental animals have established that, in cholera, antibacterial and antitoxic immunity act synergistically in protecting against disease following intestinal challenge with live vibrios. Immunization with a combination of somatic antigen (whole-cell vaccine or purified lipopolysaccharide) and toxin antigen (cholera toxin, B subunit or toxoid prepared by formalin or glutaraldehyde) gave protection that was equal to or exceeded the product of the protection induced by each immunogen alone. This suggests that combination of antigens which *per se* are capable of inducing some degree of protection might markedly enhance their protective capacity.

In the currently used killed whole-cell cholera vaccines, the main protective antigen is the lipopolysaccharide–protein complex of the Inaba and the lipopolysaccharide of the Ogawa organisms. On live cholera vibrios, however, there may exist additional surface antigens against which protective antibodies could be raised in order to interfere with the complex colonization process. Such antigens may include flagellin, flagellar sheath and as yet largely unidentified adhesive or chemotactic structures. It is also possible that antibodies to extracellular products like mucinase and neuraminidase could contribute to protection. An important protective role of antitoxic immunity is indicated from animal experiments in which immunization with highly purified toxin antigens has given very substantial protection against challenge with live cholera vibrios as well as preformed toxin. Cholera toxin is an unusually effective immunogen in that it has inherent adjuvant activities residing in its ability to stimulate adenylate cyclase. For oral immunization it also has an advantage over most other antigens in being able to bind avidly to the intestinal epithelium. It is highly unlikely that any toxin can retain the adjuvant property when it is converted into a toxoid. The best candidate for a non-living toxin-derived immunogen, especially for oral use, is probably the purified B subunit. It contains the major protective antigenic determinants of the toxin, binds as well as the toxin to intestinal membrane, and has no risk of reverting to toxicity since it lacks the toxic effector, subunit A. Immunization of rabbits with purified B subunits has given promising results.

For the development of protective immunogens against ETEC disease, it is important to identify the antigens responsible for the good protection against homologous rechallenge observed in volunteers after clinical disease. The immunity appears to be antibacterial rather than antitoxic and could conceivably be mediated by any combination of antibodies interfering with bacterial chemotactic attraction to the mucosa, penetration of mucus, adherence to brush border and multiplication *in situ*. In view of the many serotypes of *E. coli*, it is probably imperative for vaccine development to identify one or more widely shared somatic antigens on ETEC. An important shared ETEC antigen is obviously LT, which is structurally closely related but not identical to cholera toxin. The B counterpart of LT is therefore an attractive candidate immunogen for inclusion in a killed ETEC vaccine. It is also possible that the cross-reactivity between LT and cholera toxin is sufficient for the cholera toxin B subunits to confer some degree of protection also against ETEC disease, especially in persons primed with LT via natural exposure.

In both cholera and ETEC diarrhoea a live nonpathogenic, oral vaccine might represent the ideal immunogen. Such vaccine strains must retain colonization ability and should possess as many protective antigens as possible, except, of course, active toxin. Such live vaccines could be either naturally occurring nonpathogenic strains or those obtained by means of various genetic manoeuvres.

TYPHOID VACCINES

There is no reliable animal model or *in vitro* test that can predict the degree of protection afforded by typhoid vaccines in man. In controlled field trials there has been a correlation between the level of H antibodies and the degree of protection; in fact, in one recent field trial with an H-antigen-free vaccine, no protection was observed. Thus, H antigen may have a role in protection. However, it is possible that the H antigen is only a marker for a heat-labile, not yet detected "protective antigen".

Parenteral vaccines

Whole-cell vaccines. Controlled field trials in adults in endemic areas have given the following results: acetone-inactivated vaccine (2 doses)—70–85% protection for 3–4 years; heat-phenol-inactivated vaccine (2 doses)—70–75% protection for about 3 years; alcohol-inactivated vaccine (2 doses)—about 60% protection for 1–2 years. Thus, these typhoid vaccines may be beneficial for use in highly endemic areas. However, the use of typhoid–paratyphoid A and B (TAB) vaccines should be discontinued for the following reasons: (1) the incidence of paratyphoid A and B disease is generally low; (2) the immunogenicity of the paratyphoid A vaccine in man has never been proved in a controlled field trial; and (3) the results of controlled field trials have clearly shown that at least 7.5×10^8 of *Salmonella paratyphi* B organisms should be given to obtain a significant degree of protection; if this amount of *S. paratyphi* B were added to the typhoid vaccine (containing 1×10^9 *S. typhi*), the reactivity of the combined vaccine would be unacceptable.

Chemical, cell-free vaccines (Boivin, Raistrick-Topley, and Westphal types). The results obtained in controlled field trials with the vaccines have shown considerable variation in protection; generally these were less effective than whole-cell vaccines.

Oral vaccines

Inactivated vaccines. Such vaccines have been used for a long time in several countries, but their effectiveness in man has not been demonstrated. In field trials in Chile contradictory results were obtained and in 3 field trials in India no protection was found. In volunteers, some protection could be demonstrated with high, repeated doses.

Live vaccines.

(*a*) *Streptomycin-dependent vaccine.* A streptomycin-dependent vaccine has been developed that has proved to be safe in volunteers, but its efficacy has been variable.

(*b*) *Galactose epimerase-less vaccine.* This vaccine lacks the enzyme UDP-4-galactose epimerase, which prevents the normal synthesis of cell wall lipopolysaccharide. When tested in volunteers, the vaccine was safe and 87% protection was obtained. A field trial of this vaccine is in progress in Egypt.

Role of cell-mediated immunity (CMI)

S. typhi is an intracellular bacterium; therefore, as with other intracellular bacteria such as *Brucella, Listeria,* and *Mycobacterium tuberculosis,* CMI plays a major role in resistance. CMI was recently demonstrated with the leukocyte migration-inhibition test at higher rates in typhoid fever patients than in persons vaccinated with heat-phenol inactivated TAB vaccine.

SHIGELLA VACCINES

S. flexneri *and* S. sonnei *vaccines*

Parenteral vaccines. The many trials that have been carried out with whole-cell and various cell-free vaccines were completely unsuccessful.

Oral vaccines.

(*a*) *Inactivated oral vaccines.* Boivin-type polyvalent vaccine (*S. flexneri* 2a, 3a, 4a, 6, and *S. sonnei*), when given in repeated doses to *Macaca mulatta* monkeys, showed significant protection against challenge with a virulent *S. flexneri* 2a strain. *S. sonnei* vaccine, containing disintegrated bacteria, was administered to children in a large (uncontrolled) field trial and a reduction in morbidity was observed. These vaccines were administered in very large and repeated doses.

(*b*) *Live oral vaccines.* Polyvalent streptomycin-dependent vaccine (*S. flexneri* 2a, 3, and *S. sonnei*) showed a significant serotype-specific protection in large-scale trials in children and adults, but the possibility of reversion could not be excluded.

The practical application of the vaccine is limited by the multiple, high doses that need to be administered (with bicarbonate) to obtain protection. A new generation of oral attenuated hybrid *Shigella* vaccines (*S. flexneri* 2a and *S. dysenteriae* 1 attenuated by incorporation of *E. coli* chromosome; *E. coli* bearing surface antigens of *S. flexneri*) have been

tested in volunteers. The vaccines were well tolerated, but no protection could be demonstrated.

S. dysenteriae *1 vaccine.*

S. dysenteriae 1 recently caused large epidemics in Central America and parts of South-East Asia with a fairly high fatality rate; the systemic complications were especially severe.

Parenteral injection of Shiga toxoid has recently been investigated in monkeys; there was no protection against live oral challenge. No other vaccines against *S. dysenteriae* 1 have been tested.

VACCINES AGAINST VIRAL DIARRHOEAS

Present status of knowledge

Important advances have recently been made in our understanding of the etiology of acute infectious nonbacterial gastroenteritis by the discovery of two new groups of agents associated with this syndrome. The first group, of which the 27-nm Norwalk particle is a prototype, has been associated with outbreaks in school, community, or family settings, affecting chiefly children of school age and adults. Illness caused by this group is usually self-limited, lasting approximately 24–48 hours. The incubation period is characteristically about 24–48 hours. Antibody prevalence studies in residents of the USA measured by an immune adherence haemagglutination assay (IAHA) showed that serum antibody against the Norwalk agent is acquired gradually so that by the fifth decade 50% of the population had antibody. Studies of the Norwalk group of agents have been hampered by the inability to grow them in cell cultures, necessitating the use of immune or conventional electron microscopy for their detection. There appear at present to be three serotypes of this group of agents–Norwalk, Hawaii, and Ditchling agents–with the possibility of a fourth, the cockle agent. Two other similar agents, the Montgomery County (MC) and "W" agents, are related to the Norwalk and Ditchling agents, respectively. All these particles have a similar buoyant density in caesium chloride (1.37–1.41 kg/litre), the Norwalk and "W" agents are both ether-stable, and the Norwalk agent is acid-stable and relatively heat-stable. These properties, with their morphology, have led to the suggestion that the Norwalk group of agents is parvovirus-like. This is a tentative designation since it is not even known whether the nucleic acid of these agents is DNA (like the parvoviruses) or RNA.

The other, more important, form of nonbacterial gastroenteritis has been associated with infection with the 70-nm rotavirus and is characterized by a severe diarrhoea that occurs predominantly in infants and young children 6–24 months of age and may require parenteral or oral fluid therapy. The illness rate among family contacts of infants and young children with this form of gastroenteritis is low although subclinical infections occur frequently. In contrast to the Norwalk agent, rotavirus antibody is acquired early in life in a pattern similar to that for respiratory syncytial and parainfluenza type 3 viruses. Rotavirus is the major etiological agent of infantile gastroenteritis, being associated with up to 50% of hospitalized cases of diarrhoeal illness in infants and young children in many countries with temperate climates. Rotavirus infection has an unusual epidemiological feature in that it usually prevails in the cooler months of the year in temperate climates. Rotavirus disease is also prevalent in those tropical countries where it has been sought. The incubation period is about 2 days. In neonates, for unknown reasons, there is characteristically a high infection: case ratio with low morbidity. Studies of rotavirus infection in infants and young children with gastroenteritis not requiring hospitalization have been sparse; however, rotaviruses appear to play an important role in episodes of mild diarrhoea.

There are at least two serotypes of human rotavirus, designated preliminarily as type 1 and type 2. Their detection, which was initially made by electron microscopy, is now performed by numerous assay systems of which ELISA is probably the most efficient and practical.

Rotaviruses have been detected in faeces of numerous animals with diarrhoea, including calves, infant mice, foals, lambs, rabbits, deer, and antelopes, in faeces from a normal monkey, and in intestinal washings from sheep and cattle. Moreover, the administration of human rotavirus to various newborn colostrum-deprived animals induced a diarrhoeal illness in gnotobiotic calves and lambs, gnotobiotic and conventional piglets, and rhesus monkeys. Various animal rotaviruses, but not human rotavirus, have been grown very efficiently in cell culture.

Prospects for development of immunoprophylaxis for viral gastroenteritis agents

The development of a rotavirus vaccine is considered of high priority in designing a strategy for immunoprophylaxis against diarrhoeal disease of infancy and early childhood. However, there is a need first to understand the mechanisms by which

immunity to rotavirus infection is achieved. Animal studies in calves, piglets, and lambs have demonstrated the importance of intestinal antibody in preventing or ameliorating illness due to rotavirus. In especially pertinent studies in the lamb model, rotavirus antibody administered by the alimentary route was effective in inducing resistance to oral rotavirus challenge, whereas circulating antibody alone was not protective. In addition, in studies in volunteers, it appears that intestinal rotavirus IgA antibody correlates better than serum antibody levels with resistance to rotavirus challenge. Thus it would appear that antibody at the epithelial surface of the small intestine is of prime importance in resistance to rotavirus illness. Therefore, one approach to immunoprophylaxis of rotavirus infection would be the development of an oral rotavirus vaccine that would be capable of stimulating sufficient local intestinal rotavirus IgA antibody.

However, several difficulties exist in pursuing this goal. A major problem is the inability to propagate the human rotavirus efficiently in cell culture, thus at present making it impossible to produce enough human antigen for vaccine development studies. Another difficulty is the question of the duration of immunity. Adults are reinfected frequently with rotavirus but clinical illness occurs infrequently. Some provocative questions were raised by a study with Norwalk agent in which adult volunteers who developed illness on an initial challenge became ill on rechallenge 27–42 months later with the identical Norwalk strain, suggesting that immunity is of short duration. The existence of two serotypes of human rotavirus further complicates the problem of immunoprophylaxis since, at least from preliminary data, there is no cross-protection between them. Thus a rotavirus vaccine should have at least these two antigens and the possibility of other additional serotypes cannot be excluded.

Approaches to the development of a rotavirus vaccine may take several routes. One strategy might be to utilize an animal rotavirus such as the calf rotavirus to immunize human subjects if such an animal strain were able to infect humans without causing illness but still evoke protective antibodies. The human and animal rotaviruses share certain antigens but can be differentiated by tests such as analysis of RNA migration patterns by polyacrylamide gel electrophoresis and certain serological techniques. Promise for this approach was demonstrated in a calf model in which *in utero* injection of calf rotavirus protected against challenge with human rotavirus on the day of, or one day after, birth. Such vaccines would have to be tested carefully in various animal models for safety and feasibility and, if found to be safe and effective, initial human testing could take place in volunteers with preexisting high levels of rotavirus antibody (preferably IgA in intestinal fluid). Development of such a strain is being pursued.

Since the human virus does not grow efficiently enough in cell culture to even attempt vaccine development studies, production of a hybrid by genetic recombination with an animal rotavirus such as that of the calf, which grows efficiently in cell culture, might be possible. Ideally this hybrid would grow efficiently in cell culture but have the outer capsid antigens of the human rotavirus. Such a genetic approach is being pursued.

Another approach that needs further study is the use of breast milk in preventing or modifying rotavirus infection. Specific IgA rotavirus antibody has been found in human colostrum and breast milk in several populations. Rotavirus infection has been reported to occur significantly less frequently in breast-fed than in bottle-fed babies. However, the effect on illness could not be ascertained since most of the infections in both the breast-fed and bottle-fed infants were inapparent, indicating that some other mechanism was operating to ameliorate the infection. The role of breast milk in providing passive immunoprophylaxis against rotavirus infection needs further study.

In addition, the importance of rotaviruses as enteric pathogens in developing countries should be studied so that their relative contribution to the overall diarrhoeal disease incidence can be accurately assessed and their natural history ascertained on a relatively long-term basis. However, attempts to solve the problems of vaccine production should not wait for these results to be obtained since it is obvious that rotaviruses are an important, if not the major, etiological agent of diarrhoea of infants and young children.

PROMOTION AND IMPLEMENTATION OF RESEARCH

The evolving plan for implementing the research component of the diarrhoeal diseases control programme was reviewed by the working group and approved in its present form. Particular emphasis

was placed on dissemination of information concerning the mode of operation and goals of this programme, especially to developing countries through the Regional Offices, to assist in identification of scientists and institutions best suited to participate in the programme. The working group believed that institutions expressing interest in specific areas of research should be assisted in developing research protocols. It was also agreed that a uniform method was needed for evaluating research proposals. It was recommended that a proforma be developed to be used by candidate institutions in describing their research proposals.[a]

RESEARCH NEEDS

The Group felt that further research was needed in the following areas:

CHOLERA, NON-CHOLERA VIBRIO, AND ENTEROTOXIGENIC *E. COLI* (ETEC) DIARRHOEA

Protective immune mechanisms and protective antigens

There is a need for a better definition of the precise bacterial somatic structures and extracellular products that are important in pathogenesis and/or in the establishment of immunity. This knowledge will be basic to a more rational selection of candidates for development of new vaccines.

Tools should be developed for the detection of antibody against all those structures or products of the organism that play a role in pathogenesis. These would include assays of antibodies to various colonization factors involved in bacterial chemotactic attraction to the mucosa, penetration of mucus, adherence to brush border, and multiplication *in situ*. They would also include improved assays of antibodies to enterotoxins and their subunits including cholera toxin, *E. coli* LT and non-cholera vibrio toxins. Simple assays for such antibodies in serum as well as intestinal fluid are needed so that seroepidemiological studies can be carried out. There is also a clear need for an *in vitro* assay of *E. coli* ST, and for studies to define whether under certain conditions this low-molecular weight toxin might be immunogenic.

The natural course of clinical disease caused by *V. cholerae*, ETEC, and non-cholera vibrios should be determined by epidemiological studies and in volunteers with regard to:

(*a*) extent and duration of protective immunity to the homologous organism;

(*b*) cross-protection against heterologous organisms: for example, does immunity to cholera confer resistance to *E. coli* LT disease? and

(*c*) extent and duration of priming of the mucosal immune system for a secondary immune response: for example, does *E. coli* LT diarrhoea prime effectively for booster with cholera toxoids or toxin subunits?

Methods for stimulating mucosal immunity

Studies are needed of methods to initiate a protective mucosal immune response including evaluation of various antigen forms, routes of administration and adjuvants, and ways should be found to prolong both the immune response itself and the memory for response to booster. The role of each in prolonging protection should be studied.

Studies aimed at defining practical ways to measure mucosal immunity in humans are required. Such studies should include examination of extra-intestinal secretions such as saliva and breast milk to determine whether they reflect intestinal immunity. Studies should be done of antibody in breast milk to investigate its capacity to reflect intestinal immunity in the mother and to provide protective immunity to the baby, a property which might be reinforced by planned immunization of the mother.

Differences in immune responses as they relate to population differences should be investigated. This should include not only comparisons between persons in endemic and nonendemic areas but also assessment of the possible influence of age, nutritional status, genetic factors, and unrelated microbial and parasitic infections.

Development of animal models

There is a great need for a more satisfactory model than those currently available for studies of

[a] Since the meeting of the Scientific Working Group, substantial funds have been made available to WHO for research in the field of vaccine development for diarrhoeal diseases control. A proforma for use by individual research workers and institutions who wish to seek support for studies that are in line with those recommended by this Group will be available shortly and funding is expected to start in the first half of 1980. Those interested are invited to communicate with the Programnme Manager, Diarrhoeal Diseases Control Programme, WHO, Geneva, after 1 January 1980, so that the proposal form and full details of the research component of the programme may be sent to them.

pathogenesis and protective immunity in cholera and ETEC diarrhoea. The model should utilize the non-ligated bowel in intact adult animals. The establishment of such a model would greatly facilitate the pursuit of many of the studies recommended above.

Immunizing agents

Systems of genetic and biological analysis should be applied to *V. cholerae* and ETEC to provide a detailed understanding of factors associated with virulence and protection. Application of this knowledge should facilitate the rational identification of antigens that should be included in vaccines.

New and improved immunogens should be developed; these should include the following categories of materials:

(*a*) Non-living immunogens, including whole-cell vaccines, crude extracellular products and purified somatic or extracellular products such as lipopolysaccharide or toxin-derived antigens;

(*b*) Living vaccines, consisting either of naturally occurring nonpathogenic strains or laboratory-produced mutant or recombinant strains. Candidate live vaccine strains should have selective genetic markers to allow for their differentiation from wild-type strains.

There is need for continued evaluation in experimental animals, as well as in volunteers, of synergy between two or more immunogens. This will facilitate the development of combined non-living vaccines (e.g., vaccines against cholera or ETEC diarrhoea containing both somatic antigens and toxin-derived antigens such as B subunits) as well as identification of the antigens that will be required in live vaccine strains.

RECOMMENDATIONS FOR FURTHER DEVELOPMENT OF TYPHOID VACCINES

Parenteral vaccines

Since currently used vaccines are fairly reactive and do not afford complete protection, their improvement is necessary. Vaccines prepared with mild methods of inactivation, which would be immunogenic and less reactogenic, should be developed; such vaccines have been prepared recently in the USSR and their efficacy should be studied in controlled field trials. Another approach to a non-reactogenic vaccine could be to isolate the O-antigen carbohydrate structures free from toxic lipid A, or prepare these structures synthetically, and couple them to a carrier protein in order to render them immunogenic; work along these lines is in progress in Sweden. Since reactions evoked by intradermally inoculated typhoid vaccine are minimal, intradermal versus subcutaneous vaccination should be investigated in a controlled field trial. It will be necessary to compare doses as well as routes in such studies.

Inactivated oral vaccines

Although the efficacy of the inactivated oral typhoid vaccines tested so far is doubtful, further development should be continued. Preference should be given to preparations with greater antigenicity in animals. Other possible avenues of research include: (*a*) different methods of inactivation; (*b*) improved methods (schedules) of application, e.g., parenteral priming and oral boosting; and (*c*) use of adjuvants that act when given orally.

Live oral vaccines

Unfortunately, no laboratory model exists for the recognition of attenuation of virulence of *S. typhi*. Such a model should be developed. Attempts to isolate stable immunogenic strains by genetic methods should be continued.

Further efforts should be made to evaluate the new galactose-epimerase-less vaccine.

Investigation of the role of cell-mediated immunity (CMI)

It is important to develop simple methods for the testing of CMI against the typhoid bacillus. This should include investigation of the feasibility of preparing a skin-testing antigen analogous to tuberculin that could be used for detecting CMI. Candidate vaccines should be tested for their ability to induce CMI.

RECOMMENDATIONS FOR FURTHER DEVELOPMENT OF *SHIGELLA* VACCINES

Inactivated oral vaccines

Inactivated oral vaccines against shigellosis have so far been ineffective; efforts to improve methods of preparation of this type of vaccine may not deserve high priority.

Live oral vaccines

It seems that colonization, penetration, and multiplication in epithelial cells are necessary to produce disease and at the same time resistance. The present

attenuated vaccine strains do not penetrate. Perhaps, by application of genetic techniques, strains could be developed that do not penetrate, and therefore do not produce disease, but which can colonize the mucosal surface to produce enough antigenic stimulus for protection. Development of a safe and effective live *S. dysenteriae* vaccine would be particularly valuable.

The importance of *Shigella* exterotoxins in the pathogenesis of shigellosis of all serogroups needs to be better elucidated, and studies should be carried out to define the duration of intestinal immunity to natural disease.

VIRAL DIARRHOEAS

Epidemiological studies

There is a need for epidemiological—especially longitudinal—studies of the incidence of diarrhoeas caused by specific viral agents. In this way, the relative importance of the various agents can be determined and appropriate priorities established.

The impact of rotavirus disease in various populations needs to be determined and the spectrum of illnesses ascertained. Such basic information on the natural history of rotavirus infection is essential for determining which populations will benefit most from the development of a rotavirus vaccine. Such studies would help in determining (*a*) the number of serotypes and the occurrence of minor antigenic differences; (*b*) the frequency of reinfections; (*c*) the age groups most severely affected; and (*d*) the long-term importance of rotaviruses in child health.

Reference reagents for ELISA for rotavirus should be made available under WHO auspices. Greater availability of these reagents would facilitate epidemiological studies, and be of special importance in the characterization of candidate vaccine strains.

Rotavirus

Since human rotaviruses do not grow to sufficiently high titre to allow them to be used directly in a vaccine development effort, a high priority should be given to studies of methods for more efficient propagation of these agents.

Although intestinal IgA rotavirus antibody is of prime importance in preventing rotavirus illness, additional studies are needed to establish the duration and means of enhancing the immune response. Studies of rotavirus antibody levels in secretions such as saliva and breast milk are required to determine whether these reflect antibody content in small intestinal fluid.

In a disease in which local intestinal antibody plays such an important role in resistance, the low rate of illness in infected neonates is difficult to explain. The mechanism of such resistance requires study.

Longitudinal studies should be carried out to determine the role of breast feeding in the natural history of rotavirus infection. Epidemiological, immunological, and social factors should be investigated. Knowledge gained from such studies would help determine whether a rotavirus vaccine should be administered to females of child-bearing age.

Since passive administration of rotavirus antibody by the alimentary route has resulted in resistance to rotavirus challenge in various animal models, studies in man on the effect of oral administration of human rotavirus antibody might be considered. Another approach might be the oral administration of cow's "immune milk" (containing antibody to rotavirus).

An animal in which disease could be induced beyond the early period of life should be sought. This would be important for the study of the safety and efficacy of candidate rotavirus vaccines.

Norwalk group of agents

Additional efforts should be made to discover animal models for the study of illness caused by the Norwalk group of agents.

The observed absence of long-term immunity to Norwalk agent in volunteers has raised perplexing problems in connexion with intestinal immunity. The reasons for this failure need elucidation.

Other viral agents

Efforts should continue to search for, characterize, and determine the importance of other viral agents that may be associated with viral gastroenteritis.

RECOMMENDATIONS FOR GENETIC STUDIES

There is every reason to believe that recent advances in microbial genetics and DNA biochemistry can be exploited and applied to the practical problems of enteric vaccine development. This will require an appreciation of both the potential usefulness and limitations of the genetic approach. Although genetics is often viewed as a technically complicated field, in point of fact genetic crosses can

be accomplished with bacteriological media less complex than those used in the enteric diagnostic laboratory. One can readily train individuals to perform rather detailed plasmid analysis of strains using relatively inexpensive reagents and requiring no more sophisticated equipment than a home-made slab-gel apparatus and a direct current power supply. These methods have, in fact, already been taught, under WHO support, to technologists and clinicians from the developing nations and have been used to good advantage in their home countries upon their return.

Further efforts to encourage joint training and collaboration of epidemiologists, immunologists and microbiologists along with those who have genetic and molecular expertise are therefore recommended.

In addition to the genetic studies cited in the preceding sections, there is need to:

(*a*) make available a better selection and variety of well-characterized donor and recipient strains of enteric pathogens to facilitate study of genetic determinants of pathogenesis;

(*b*) improve the characterization of *Ent* plasmids from ETEC of man and their relationship to those of animals;

(*c*) study the nature and genetic basis (whether chromosomal or extrachromosomal) of colonization by human ETEC, *V. cholerae,* and other enteric pathogens; and

(*d*) determine whether plasmids contribute to the pathogenesis of salmonellosis, shigellosis, or cholera.

ESTABLISHMENT OF NEW VOLUNTEER FACILITIES IN ENDEMIC AND NONENDEMIC AREAS

Certain questions concerning pathogenesis and immunity in all of the infectious diarrhoeas can be answered conclusively only in volunteers. Thus there is a need for establishment of volunteer facilities in endemic and nonendemic areas. Such facilities should provide the possibility for studying:

(1) the response to oral challenge with living bacterial and viral agents in order to define the natural course of disease;

(2) the immune response in terms of antibodies in serum and other body fluids; and

(3) the protective value of candidate vaccines.

In all of these studies the highest ethical standards must be followed in selecting and informing volunteers and their rights must be fully respected. Volunteer study centres should be operated under the direction of local investigators. The protocols should be reviewed by local ethical review committees composed of some individuals not directly involved in the project.

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