PRINCIPLES OF ACTIVE IMMUNIZATION[1,2]

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Successful active immunization depends, in the first instance, on the existence of a pertinent immune mechanism in the disease in question. In addition, it depends on the development of an effective antigenic preparation for use in immunization against the disease, and evidence that this preparation is acceptable for use in man. Furthermore, it is now considered essential to establish, by means of soundly designed field trials, that the immunizing agent actually provides a satisfactory degree of protection. In recent years this requirement has been imposed upon vaccines that had been in use for over half a century. Thus, at last there is genuinely acceptable evidence for the efficacy of typhoid vaccine (1–6) and, to a lesser extent, for cholera vaccine (7).

Apart from such considerations, it is desirable to examine, in detail, the dynamics of the procedures that are employed in active immunization. Although there are great variations among such procedures, certain generalizations are beginning to emerge, derived both from studies on immunization and from advances in general immunology. This review will deal with a limited number of these areas of progress, where they appear to bear most directly on advances in active immunization.

Live Versus Killed Vaccines

Two widely differing approaches to active immunization have long been recognized; the use of live, attenuated strains of the disease agent in question as with smallpox vaccine, or the use of processed inactivated components of the etiologic agent, ranging from crude suspensions such as typhoid or pertussis bacilli to the refined, selected antigenic fraction in a purified diphtheria toxoid. Little critical study of the essential differences between these approaches has been done. It is apparent that a live vaccine which multiplies in the host will generally produce a relatively massive outpouring of antigen compared to what can be achieved, within the limits of host tolerance, with most killed antigens. Also, it is generally assumed (usually without adequate evidence) that the antigens in living vaccines are antigenically "closer" to those of the virulent disease-producing agent itself. In addition, it is widely believed that a live antigen engages certain tissues in some vital immunological commitment that cannot be achieved by the corresponding killed

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antigen. Occasionally, in a special case, this belief receives experimental support—as with oral poliovirus vaccine which, probably because of its route of administration, induces massive local immunity in the gastrointestinal tract, thus blocking the entrance of subsequent wild, virulent strains into the host (8). A simpler example of the differences that can arise in the pattern of the immune response to live vs. inactivated vaccines is seen in the various serologic studies carried out following immunization against measles. This has been done with live attenuated "Edmonston B" strain vaccine, with the same vaccine plus measles immune globulin, with multiple doses of killed measles vaccine, with one or more doses of killed vaccine followed by live attenuated vaccine, with the further attenuated "Schwarz" strain of live vaccine, etc. A recent study by Krugman et al. (9) demonstrates that the serologic response to the Edmonston B strain vaccine, as measured by the long-lasting HI or neutralizing antibodies, shows scarcely more than a fourfold drop from peak titer, even after four years have passed. By contrast, Rauh & Schmidt's subjects, injected with three doses of killed measles vaccine at one-month intervals, showed not only a considerably lower peak titer but what appears to be a relatively greater drop in titer with time (10). Krugman et al. (9) also observed that the level of the antibody response fell off more rapidly after injection of live vaccine if preceded by two doses of killed vaccine. Similarly, varying degrees of accelerated decline in the antibody level with time were seen if gamma globulin was given simultaneously with either Edmonston B or "further attenuated" live vaccine. These findings would be consistent with the supposition that the nature, extent, and duration of an immune response are largely dependent upon the mass of the initial antigenic stimulus, the sequence of succeeding stimuli, and the presence and concentration of antibody during the various stages of the response. However, other studies have shown that measles virus invasion can occur in the presence of significant levels of measles neutralizing antibodies induced by immunization with killed vaccine, whereas very low titers of antibody induced by natural infection or with injection of live vaccine appear to prevent invasion by the measles virus (11). Thus, there may be qualitative as well as quantitative differences in the responses to killed vs. live vaccines.

The Initial or Primary Immune Response

Studies on the immune response using live antigens cannot be made quantitative, since the amount and duration of the multiplication of the agent are difficult to determine and even more difficult to control. Therefore precise studies of the immune response have generally been carried out with the use of nonviable antigenic substances—usually a relatively "pure" antigen such as bovine serum albumin, bovine gamma globulin, diphtheria toxoid, tetanus toxoid, etc. Within a few hours after the first injection of such an antigen, demonstrable antibody formation may have begun (12, 13), and the steps preceding this manifestation must clearly have begun even earlier. Fishman (14) has provided evidence that the initial step is phagocytosis of the antigen. A cell-free homogenate of phagocytes, prepared after they were

incubated with the antigen, was capable of stimulating plasmacytic cells to form the corresponding antibody. The activity resided in the RNA fraction, and ribonuclease treatment of the homogenate rendered it ineffective (14, 15). This does not necessarily mean that the antibody-forming stimulus at this point is mediated by a messenger-type RNA, for Askonas & Rhoades (16) have found traces of antigen present in such extracts, and they suggest that RNA may simply enhance the uptake of antigenic material (whatever that may be at this stage) by the relevant cells. Although some studies suggest that more than one "relevant" cell type may be involved, this question will be difficult to answer until more general agreement is reached on the relationship between the various mononuclear cells that have been implicated, the terminology to be employed, and the parallelism of observations made from divergent starting points and with divergent techniques. The complexity of the problem is well brought out in Yoffey's review on "The Lymphocyte" (17).

The first serologic response to a protein antigen (12, 13, 18, 19) appears to be the formation of a large macroglobulin, with a sedimentation constant of about 19S, formerly called "gamma 1-M" or "beta 2-M," now generally known as gamma-M globulin or IGM (20). The formation of IGM proceeds actively for several days and then appears to subside almost as fast as it developed. It appears from the observations of Jerne et al. (21), Koros et al. (22), and many others that the antibody-forming cells proliferate vigorously in the first few days following antigenic stimulus. The extent of this response is related—as one might expect—to the number of such cells activated (23) and the extent of their proliferation which may perhaps be, under some circumstances, as great as 1000-fold (21).

Meanwhile, if the antigenic stimulus was sufficient, another, smaller type of antibody globulin appears, with a 7S sedimentation constant and known as gamma-G globulin, IGG, or formerly gamma 2-globulin (20). Production of IGG is sustained for a relatively long period of time. However, with small doses of antigen, only the IGM type of response may be induced (12, 13) and successive small doses of antigen, at suitable intervals, may induce successive similar transient IGM responses (24). The antigen dose required for the induction of IGG was, in Svehag's system, about 50 times that which sufficed to induce IGM antibody.

Essentially similar patterns have been observed following experimental infections (25). On the other hand, the response to the injection of lipopolysaccharide antigens consists essentially of IGM (18), which remains predominant even after repeated antigenic stimuli (26). It has been reported that such antibody responses (19) as well as those to Vi antigen (27) or pneumococcus carbohydrates (28) remain at high levels for many months. Others have not observed such a sustained response (29). However, it is difficult to determine whether sustained responses to this class of antigens are actually primary in nature. The difference between these two patterns of response has intrigued many investigators, and may have more than academic importance. On the one hand, IGM participates much more efficiently than IGG in the

immune hemolysis of erythrocytes (30); indeed, the relative efficiency of IGM in this respect is on the order of 750-fold (31), and a comparable difference has been observed with respect to immune opsonization of bacteria (32). On the other hand, IGM appears to be very much less efficient than IGG in neutralizing diphtheria toxin (33). Thus, the differences in the behavior of IGM and IGG antibodies may bear not only on the measurement of antibody responses but on the clinical significance of immunization procedures.

The production of IGG appears to have a suppressive effect on the production of the corresponding IGM (34, 35, 36). In addition to this immunospecific mechanism there appears to be a broader feedback system whereby increased levels of each type of gamma globulin lead to accelerated catabolism of that type (37). Thus, the formation of gamma globulins may well be subject to one or more mechanisms of homeostatic control.

Nature of the Antigen

Most vaccines, as now employed, contain many antigens, of which only one, or at the most a few, may be essential for the protective immunity desired. In a few instances protein antigens (e.g., diphtheria and tetanus toxins and toxoids), polysaccharide antigens (e.g., several of the pneumococcus capsular substances), and lipopolysaccharide antigens (e.g., the cell wall or "O" antigen of the typhoid bacillus) have been isolated and characterized to a greater or lesser extent as purified entities. From the point of view of immunization, the central issue in each case is: what makes an antigen immunogenic? A vast amount of work on the polysaccharide and lipopolysaccharide antigens has led in many instances to identification of the structural portion of the molecule which determines its antigenicity. For polypeptide and protein molecules, the problem has proved more difficult, and is being approached with more promise of success through the synthesis of artificial polyaminoacid antigens. Several recent reviews cover this highly specialized subject well (38, 39, 40). It appears that not only the number but the variety, the internal structural relations, and the specific identity of the amino acids incorporated into a polypeptide antigen have a major influence on its "antigenicity." Meanwhile, the reasons for the differences between natural antigens such as diphtheria and tetanus toxoids—the latter being apparently much the more efficient of the two in terms of antibody produced per microgram of antigen injected—remain unexplained but nevertheless important in designing immunization reagents and procedures.

Amount of Antigen

Many years ago, Smith & St. John-Brooks (41) showed that a clear mathematical relation could be established between antigen dose and peak response obtained, and pointed out that their mathematical expression was similar to the so-called Freundlich adsorption isotherm equation. A decade ago Stevens revived this finding (42), tested it against thirteen sets of published data on presumably primary immunization of man or animals, and five sets of data on the secondary response. The equation gave a good fit, and other sets of data have subsequently been fitted to this logarithmic straight-

line equation (43). Although the aberrant dose-response curves published by Stille (44), together with some analyses of existing data by Gottlieb et al. (45), raise some doubts as to the extent to which the "straight line" assumption should be accepted, neither set of observations in any degree alters the fact that the amount of antigen employed plays a major role in determining the extent of the immune response.

Whether this dose-response relationship is more significant for the primary than the secondary response is not clearly settled. Ipsen's findings (46) suggested that the dependence of response on dose was greater in the primary than in the second-dose response in man. However, the data assembled by Stevens (42, 43) as well as the experimental findings of Uhr, Finkelstein & Baumann (13) do not indicate a significant difference in this respect. On the other hand, it is clear as noted above (12, 13) that a certain "threshold" amount of antigen must be incorporated in the primary immunizing dose, or else the reaction to the next dose will be in essence another primary response. What this "threshold" is will, of course, vary from antigen to antigen. The small amounts of antigen in the usual Salk vaccine (47), comparable on a weight basis to 1 Lf or less of diphtheria or tetanus toxoid, may have reflected such a marginal level. Schluederberg's observation (48) that, after a second dose of killed measles vaccine one frequently sees a 19S (IGM) response, is probably another instance of marginal antigenic mass.

State of Aggregation of the Antigen

Winebright & Fitch (49, 50) showed that flagella particulates or intact flagella of *Salmonella typhimurium* induced a more rapid antibody rise (irrespective of the route of injection) than was seen with soluble flagellar antigen, and Gamble (51) likewise showed that aggregated human gamma globulin was more immunogenic than its monomeric form. Indeed, Dresser (52), confirmed by Claman (53), has shown that certain antigens may be antigenic in the aggregated form but may actually induce the reverse response—immunologic tolerance or "immunoparalysis"—if injected, even in small quantities, in the soluble, nonaggregated form. These findings doubtless shed light on the reported low antigenicity of highly purified diphtheria toxoid (54).

Other data supporting the assumption that aggregation of an antigen favors its efficacy date from the old observation that diphtheria toxin, in quantities insufficient to be antigenic by itself, was markedly antigenic when combined in optimal proportions with diphtheria antitoxin. More recent findings such as those of Terres & Wolins (55) and of Segre & Kaeberle (56) attest to the enhancing effect of traces of antibody, *in vivo*, in inducing primary antibody formation.

All of these observations can be tentatively reconciled with the concept that aggregates of an antigen are more readily phagocytosed than the antigen in solution, that phagocytosis is an essential step to antibody formation (or at least, to efficient antibody formation) and that the more antigen is phagocytosed, the more antibody formation will result, other factors being equal.

Adjuvants

Many chemically unrelated substances have been identified as immunological adjuvants—i.e., under suitable circumstances they enhance the immune response. Three principal types of adjuvants concern us here: aluminum salts, water-in-oil emulsions, and endotoxins. The adjuvant effect of aluminum salts has been known for nearly half a century. They have been shown to enhance the primary immune response to toxoids somewhat in man (57) and markedly in guinea pigs. In many other studies their usefulness in enhancing various other antigens [e.g., poliomyelitis vaccine (58), pertussis vaccine (59)], has been established. This effect may well be—as seen with tetanus toxoid in Eckmann's studies (60)—based primarily on the enhancement achieved by alum with the initial dose. It is easy to assume that the adjuvant effect of aluminum salts is due to their enhancing effect on phagocytosis of the antigen; but this assumption remains to be proved. Furthermore, there is little evidence that aluminum salts enhance the immunogenicity of polysaccharide antigens.

Water-in-oil emulsions (e.g., the so-called "incomplete Freund adjuvant") greatly enhance the primary immune response, both in titer and in persistence of high antibody levels. Such emulsions, the efficacy of which was pointed out over 20 years ago (61), do not apparently alter the rate of primary antibody formation, but they induce a more prolonged and hence much higher rise in titer (62, 63). In addition, these high titers generally persist for exceptionally long periods of time, in contrast to the early asymptotic decline of antibody levels after injection of the same antigen in the usual fluid state. An interesting comparison of the responses to fluid, aluminum salt-adsorbed, and water-in-oil emulsified antigens has recently been completed by MacLennan et al. (64) who studied the response to tetanus toxoid prepared, in the three ways indicated, from the same batch and in the same amount per dose. Two doses of the aluminum preparation induced about twice the average peak response, and five to ten times the one-year level, as compared with three doses of the fluid toxoid; a single dose of emulsified toxoid not only gave a peak titer higher than that seen with the aluminum preparation, but induced a level one year later which was 20 to 30 times higher than that found after the injection of three doses of fluid toxoid.

The mechanism of the enhancement of antigenicity by water-in-oil emulsions is not clear, especially since this procedure also offers no detectable advantage with polysaccharide antigens. It has been suggested that it mobilizes phagocytes to the site of antigen deposition, and that it leads to prolonged retention of antigen at the various sites where the antigen-containing emulsion is ultimately deposited. Regardless of the mechanism involved, it is clear from the demonstrated long persistence of antigen, that the stimulus provided by this technique is not a pure "primary" stimulus but a complex of primary, secondary, and perhaps recurring primary stimuli. Thus, the place of the water-in-oil technique in immunization procedures is technically not easy to define.

Controversy still surrounds the practical application of the water-in-oil technique in man. Although Beebe et al. (65) found no evidence of long-term

side effects attributable to the use of water-in-oil influenza vaccines in several thousand United States military recruits, and a Medical Research Council study (66) found a similar vaccine acceptable and antigenically very effective in a civilian population of varied ages, nevertheless, several unpublished reports indicate that unforeseen high reaction rates occur from time to time with such preparations, perhaps especially when used with bacterial vaccines.[3] Therefore each proposed application of this technique needs to be individually and critically studied before its acceptability can be clearly estimated.

Endotoxins.—Endotoxic substances appear to have an adjuvant effect of a fundamentally different nature. A decade ago Johnson et al. (67) showed that the "O" or "cell wall" antigen of *Salmonella typhosa*—a typical and classic endotoxic substance—enhanced the response to a protein (but not to a lipopolysaccharide) antigen when injected at more or less the same time as the antigen. This apparently explains the long recognized enhancement of the antibody response to a toxoid if it is given as a mixture with typhoid vaccine (68) or pertussis vaccine (69). Zweifach & Janoff (70) and Johnson (71) have reviewed the complicated factors underlying this adjuvant system, including its limitations. Several studies on the adjuvant mechanism point to the conclusion that it depends on the liberation of nucleic acid breakdown products (72, 73, 74). Merritt & Johnson (75) have shown that the empirical effect on antibody formation is apparently to shorten the induction period; however, the mechanism whereby the onset of detectable antibody formation is thus accelerated remains a matter of speculation (74).

The Secondary or Booster Response

When protein antigen is administered in sufficient quantity to produce IGG, as noted above, it alters the reaction of the host to that antigen, so that a subsequent injection of the antigen will induce an antibody rise consisting almost entirely of IGG, and the peak level reached (given the same antigen dose) will be higher and sometimes earlier than after the primary response. The level will fall rapidly thereafter, but will approach an asymptote at a higher level than existed prior to the second dose of antigen. This is the typical "booster" response, seen in innumerable studies with toxoids in man, or with various protein antigens in rabbits, etc. This response appears to depend on the retention of what is conveniently called "immunological memory," apparently by descendants of cells that were involved in the IGG response after the first contact with the antigen in question.

When a secondary response is initiated, mitosis takes place in those lymphatic plasmacytes which are apparently both the descendants of the primary-response cells, and the precursors of the mature cells producing IGG in the full-blown secondary response. Mitosis continues—in the rabbit, for example—at a peak rate which may be as great as every six to seven hours, for about eight generations, after which it terminates almost as abruptly as it

[3] A striking example of such reactions has recently been reported following the use of a water-in-oil emulsion of cholera vaccine (Philippine Cholera Committee, *Bull. World Health Organ.*, **32**, 603–25, 1965).

began (76). Thus, the antibody-forming potential, following a secondary response, may increase well over 100-fold in a few days. Allowing for the many variables that must affect the outcome, this figure is not inconsistent with the finding of Gottlieb et al. (77) that the tetanus booster response in man centered around a general rise of about 70-fold, but varying in accordance with several other influencing factors. The secondary response to lipopolysaccharide cell wall antigens has not been studied in as much detail. As noted above, lipopolysaccharide antigens induce antibody responses almost entirely of the IGM type. Only with repeated inoculations does a small amount of IGG antibody develop (26, 78). What this signifies for immunization preparations or practices is not yet clearly definable; however, the long duration of the immune responses to pneumococcus carbohydrates (28) or Vi antigen (27) suggests that for some immunization procedures the booster mechanism may play a relatively minor role. The growing evidence (2, 3, 5) that single doses of typhoid vaccine appear to give a high and relatively durable level of protection against typhoid fever may conceivably prove to be a related phenomenon.

The secondary response is apparently enhanced by adjuvants, although perhaps less than is the case for the primary response (60, 62). Eckmann found that the use of aluminum salts as an adjuvant for a primary injection of tetanus toxoid greatly enhanced the subsequent secondary response to either form of the antigen.

The timing of secondary doses of antigen appears to be critical with respect to the minimum interval, whereas there is no clear evidence for the existence of a maximum effective interval. Many years ago Carlinfanti (79) showed that successive daily injections of antigen were more efficient than the same amount of antigen given as a single dose. However, numerous serological studies in man support the general belief that for most antigens the response will be impaired if the interval between doses is less than about three to four weeks; there are no precise data applicable to this effect in man. Fecsik, Butler & Coons (80) showed an increase in the response of mice to a second dose of diphtheria toxoid as the interval increased from 10 to 40 days, and no further enhancement with intervals up to 180 days.

Within a narrower time range Brown et al. (81) found, in three comparisons of one- vs. two-month intervals between injections of DPT-Polio vaccine, that the response to the longer interval was invariably superior.

Maintenance of Immunity

Following most immunization procedures, the measurable antibody level falls slowly, generally toward an asymptotic level which varies from one individual to the next. In a number of instances it has been shown that clinical susceptibility develops more or less in parallel to this fall in serologic immunity. Therefore periodic "booster" doses have been employed with essentially all immunization procedures. The earlier tendency to schedule booster doses at relatively short intervals has been subject to progressive revision, as evidence accumulated that the antibody level either fell off extremely slowly in later years (77) or in some cases appeared to remain unchanged for years

after the initial post-booster drop (82). Furthermore, the capability of responding to a secondary antigenic stimulus has been demonstrated to last for over 20 years with tetanus toxoid (77), 25 or more years with rabies vaccine (83), over 10 years with typhoid vaccine (84), and over 15 years with yellow fever vaccine (85). In fact, such long persistence of immunological memory appears to have been found wherever it has been sought.

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