PN-AAU-856
ISN 23168
B 9310453006301

Reprinted from: IMMUNITY TO PARASITIC ANIMALS,
Volume 2

Edited by: G. J. Jackson, R. Herman, and I. Singer

Published by Appleton-Century-Crofts, New York,
Educational Division, Meredith Corporation

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INTRODUCTION

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Vaccination: Progress and Problems

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INTRODUCTION

Prophylactic immunization as a means of controlling parasitic infections in man and his domestic animals has not, until recently, played a major role in veterinary or medical practice, nor in the planning of research programs which were aimed at the development of control measures. For example, the

relationship of the macroscopic helminths to disease has been known since recorded history, yet the recognition of resistance to infection and the application of this finding in attempts to induce protection against these parasites is only recent.

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This situation contrasts strongly with the early history of bacteriology and virology. Thucydides (460–404 B.C.), the historian of the Peloponnesian War, commented on the well-known fact that survivors of the plague were never known to suffer from the disease again and were important as nurses to the ill. Smallpox vaccination traces its history to early observations in Asia that survivors of childhood infection remained unaffected during subsequent recurrent epidemics. The introduction of heterologous cowpox vaccine as a safe and effective human immunizing agent by Jenner (*circa* 1796) is well-known. Finally, the development of vaccines against anthrax and rabies by Pasteur and against diphtheria by Roux and Yersin in the latter part of the nineteenth century followed very shortly the isolation or demonstration of an infectious organism as the causative disease agent.

The only similar situation in the field of parasitic diseases is that of dermal leishmaniasis (Oriental sore) caused by the protozoan, *Leishmania tropica*. It has long been known that people who recovered from a case of Oriental sore never developed another. For this reason, the practice became established of inoculating, by scarification, material from an active sore into a site in children (particularly females) where the cosmetic effects would be less noticeable.

In the central Asian republics of the U.S.S.R., prophylactic vaccination is widely practiced, using material obtained from cultures of a zoonotic strain of *L. tropica* isolated from wild gerbils (*see* Manson-Bahr, 1963; Stauber 1963). Unfortunately, very little detailed information on the preparation and effectiveness of this living vaccine is available.

One of the earliest reports of parasitic immunity observed under laboratory conditions is that of Theobald Smith (1910), who recognized the development of immunity to the sporozoan protozoa, *Eimeria stiedae*, in rabbits. Since Smith's report, the research on the mechanisms of resistance to coccidiosis, which has included numerous attempts to stimulate resistance by means of dead antigenic material prepared from various stages in the life cycle of the parasite, has not been successful. Active immunity in avian and rabbit coccidiosis, as in the case of the leishmanias, has been produced only through the use of living material (Horton-Smith et al., 1963).

Although the use of dead material was equally unsuccessful in early attempts to confer resistance to trypanosomiasis, it was eventually found that such antigenic preparations could be used to stimulate an apparent sterile immunity (Soltys, 1964). Soltys found that:

> Success in immunizing rabbits, rats, and sheep with dead trypanosomes depends on the method of killing trypanosomes, the frequency of inoculations and the strain used for immunization. Positive results were obtained when trypanosomes were killed by formalin or by freezing and thawing five times. Negative results were obtained when trypanosomes were killed by 56°C for half an hour.

This statement on the quality of antigen and frequency of vaccination may well be applied to all the parasitic vaccination studies which have been undertaken with dead antigens. It underlines the importance of testing a wide variety of experimental immunization procedures and antigen preparation methods before negative results are conclusively accepted.

In the case of malaria, the most important protozoan parasite of man, acquired immunity and the possibility of prophylactic immunization are now being seriously considered (Sadun et al., 1966; Goodman, 1966). In areas of hyperendemic malaria, the necessity of several years of exposure to constant reinfection in order to acquire a level of resistance sufficient to suppress acute manifestations of the disease suggests formidable practical problems in the development of a vaccine (Cohen and McGregor, 1963). Nevertheless, encouraging results by Targett and Fulton (1965) with trophozoite antigens of simian malaria, Richards (1966) with sporozoite antigens of avian malaria, and the serotherapeutic success of Cohen and McGregor (1963) using concentrated gamma-globulin to treat children with high parasitemias of *Plasmodium falciparum* led workers to give greater significance to the immunologic response to this infection and its possible use in control. With the development of drug resistance by the plasmodia and insecticide resistance by the mosquito vectors, the need for an immunologic approach is further emphasized.

Among the first workers to recognize the phenomenon of acquired resistance to helminths was Stoll (1928, 1929, 1958). His discovery of acquired immunity in sheep to the stomach worm, *Haemonchus contortus*, along with his suggestion that it was similar to the resistance following bacterial infections, did not gain immediate acceptance by parasitologists. Today the picture is quite different. Stoll (1961) openly and rhetorically asked, "The worms: can we vaccinate against them?" His qualified affirmative answer is based largely on the work carried out with parasitic nematodes.

Jarrett et al. (1959) introduced the first commercially produced vaccine against *Dictyocaulus viviparus*, the lung worm of cattle. The vaccine, which consists of living infective larvae attenuated by ionizing irradiation, has been in use since 1959 and has proved to be safe and effective (Poynter and Terry, 1966). However, this technique has not been found to be so successful when applied to other helminth species. The reasons for this are not clearly understood, but the inconsistency in results has emphasized the needs for a better understanding of the mechanism of resistance and for the isolation of the functional antigens involved. Progress toward this aim has been slow but encouraging (Silverman, 1965a).

Resistance to the larval stages of tapeworms was first described by Miller (1931) and Campbell (1936). The role of functional antigens associated with different stages and different activities is strongly confirmed by the recent work of Gemmel (1965a,b, 1966), who investigated the use of heterologous activated hexacanth embryos as a living vaccine in sheep against *Echinococcus granulosus*. The importance of transitory antigens associated with early penetration and establishment of the parasite is also apparent from studies

with schistosomes, the blood fluke of man and domestic animals (*see* Sad 1963; Silverman, 1966).

ACTIVE IMMUNIZATION

The details of specific host-parasite relationships and the present status studies on immunity to protozoa and helminths are summarized in t preceding chapters. It is the purpose of this contribution to deal with some the factors which are *common* to the process of prophylactic immunizatio My thesis is that vaccination procedures, either alone or in association wi biologic or chemotherapeutic measures, can make a useful and significar contribution to the control and treatment of parasitic infections. Further more, as already indicated, it is becoming increasingly clear that protozoa and metazoan parasites stimulate the same kinds of immune reactions tha are known to occur in response to bacteria and viruses. The hypothesis tha unless natural infection readily induces resistance, it is not possible to stimu late it artificially, no longer appears tenable when one considers parasiti infections.

Many of the puzzling phenomena concerning the apparent lack of immu nity to natural infection with parasites can be shown to be due to the chan ing antigenic character of metamorphosing parasites, to subthreshold stim lation by transitory functional antigens, or to other factors associated wi the complex nature of parasitic infections. The research challenge is t unraveling of the qualitative and quantitative mechanisms by which recogn tion of and resistance to parasites can be enhanced through artificial or natu ral means. The pessimistic promulgations which have been broadcast o this subject are inevitably based on negative data. As Schad (1966) ha pointed out, negative data do not rule out immunologic explanations in th case of inhibiting interactions between heterologous parasites. Neither d they rule out interactions between host and parasite.

ANTIGENS

Stage-specific antigens

Protozoa. The results of passive transfer of gamma-globulin to parasitized children from adults with many years of exposure to malaria in an hyperendemic area suggest that immunoglobulin "may act upon mature intracellular parasites or inactivate merozoites liberated from red blood cells" (Cohen and McGregor, 1963). Subsequent to passive transfer of protective sera, the rapid fall in trophozoites after schizogony is followed by an increa in gametocytes which appear to be unaffected by immune sera.

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Richards (1966), interested in active immunization against malaria, used sporozoite antigens of avian species. He observed that immunized animals which were completely resistant to challenge with sporozoites could still be infected by the erythrocytic stages when these were injected directly into the blood stream.

The observations of Cohen and McGregor (1963) and Richards (1966) are illustrative of a phenomenon characteristic of parasitic infections, i.e., the diversity of antigenicity associated with various stages of a parasite occurring in the same host. The fact that immunity develops naturally only after many years of exposure suggests that the replicating blood stages are poor antigens in stimulating resistance to reinfection. The highly effective immunity obtained experimentally by Richards (1966) with sporozoites raises the question of whether the minimal exposure in nature (apparently less than 30 minutes) of this immunogenic stage is below the immunologic threshold required for the stimulation of active immunity in the host.

Evidence of stage-specific antigens in the case of fowl coccidiosis has been reported by Horton-Smith and his co-workers (1963). They found that second-generation merozoites and the subsequent gametocytes were poor in their ability to stimulate resistance to reinfection with challenge doses of second-generation merozoites. After three or four repeated infections with second-generation merozoites, the birds become resistant, but were shown to be completely susceptible to oral challenge with oocysts. No evidence of protection was found against schizogony or gametogony. It was concluded that exposure to both the sporozoites and schizogony were necessary in the natural stimulation of resistance. In both fowls and rabbits, the primary and probably most important immunologic barrier to parasite development appears to be at the sporozoite stage. Schizogony is seemingly suppressed entirely by the host's resistant response after development of immunity.

Helminths. The immunogenic stages which have been identified as important sources of functional antigen (i.e., resistance stimulating) in natural helminth infections elaborate substances during early infection and development of the metazoan parasite (*see* Silverman, 1965a, 1966).

Infection with parasitic nematodes is initiated by second- or third-stage infective larvae. In animals which have developed resistance as a result of repeated exposure to reinfection with third-stage larvae, the stage at which resistance is directed is the molting fourth-stage larva and, to a lesser extent, the molting fifth-stage. The adult worm does not appear to be as important as a source of resistance-stimulating antigen nor to be as adversely affected by the resistance response as the larvae. For this reason, animals which are resistant to *reinfection* may still maintain a worm burden of adult stages.

The existence of stage-specific antigens raises a number of important problems from the viewpoint of helminth vaccination and helps to explain the failure of some vaccination attempts which utilized the most readily available, but inappropriate, adult worm antigens. It appears that functional antigens may be associated with the somatic tissues of certain larval stages or may be secreted or excreted as toxins or metabolic products. To date, such

antigens have been obtained only from actively metabolizing parasites. After the parasite stage(s) responsible for elaborating the resistance-stimulating antigens have been determined, techniques for collecting and standardizing the appropriate antigens must be developed.

Strain-specific antigens

Although much emphasis has been placed on evidence of strain- and species-specific immunity, a growing body of data suggests that there are many biologically important cross-reactions and basic species antigens that transcend strain variations. Gray (1965), in an extensive study of *Trypanosoma brucei* transmitted by two species of tsetse flies to goats and rabbits, followed the development of serologic substrains. He found that a common basic strain antigen persisted throughout the variants, and the trypanosomes eventually reverted to the basic strain in the absence of antibody, i.e., in the invertebrate host. Brown and Brown (1965) observed a similar phenomenon with monkey malaria.

Of even greater interest are the experimental findings of Nussenzweig et al. (1966), who induced a high degree of protection in mice to *Plasmodium vinckei* by prior infection with *P. chabaudi*. Voller et al. (1966) have also reported on cross-immunity between several species of monkey malaria. In contrast, the coccidia remain distinguished by their apparently high degree of species specificity (Horton-Smith et al., 1963).

Among the helminths, numerous examples of immune cross-reactions between species have been reported, and no evidence of intraspecific strain specificity has been reported. Although research workers have postulated strain differences to explain failures to reproduce experimental results in different laboratories by different workers using the same helminth species, other explanations seem more overriding. The age and sex of host animals, size of inoculum, site and route of immunizing injections, and type of adjuvant have varied greatly and could account for all the differences which have been described. Strain differences of immunogenic importance might exist amongst helminths, but the evidence to date is inconclusive.

The ability of some phylogenetically widely separated species to induce protective reactions (*see* Schad, 1966) is illustrated in the work of Silverman et al. (1962), who found that antigens prepared from *Haemonchus contortus* stimulated a highly significant degree of resistance in guinea pigs to infection with *Dictyocaulus viviparus*. On the other hand, *H. contortus* antigen had no apparent effect in protecting guinea pigs when challenged with *Trichostrongylus colubriformis*. The inability to predict where and how the useful antigens for potential vaccines might be obtained should encourage research workers to cast their nets widely. In our laboratory, Jakstys (1969) has found serologic cross-reactions between antigens of the free-living nematode, *Caenorhabditis briggsae*, and the parasitic nematode, *Haemonchus contortus*. Lehnert (1967) also has demonstrated similar reactions between *Ascaris suum* and *C. briggsae*.

In many invertebrates, numerous activities such as molting have been found to have common physiologic and biochemical mechanisms. Molting hormones in the class Insecta can be prepared from one order of insects and shown to be active in all respects in other insect orders. In those cases where chemical characterization of insect hormones has been completed, little difference has been found when distinct insect species are used as sources of hormone. If molting fluids of helminths are important functional antigens, there is no reason to believe that they will be species-specific, since specificity seems to offer little selective value if the analogy with insect hormones is valid.

It is obviously too much to expect that a single nonspecific antigen will be found that is active in stimulating protection against a whole spectrum of parasite species; on the other hand, there is enough presumptive evidence so that the concept of common antigens within limited groups might have useful applications.

Living organisms

Immunizing procedures for parasitic infections have been investigated using living (homologous and heterologous) organisms, attenuated organisms, and dead antigen vaccines.

Although living organisms have been used to vaccinate against certain parasitic diseases, e.g., fowl coccidiosis, human dermal leishmaniasis, monkey schistosomiasis, and bovine lung worm (Jarrett et al., 1959; Manson-Bahr, 1963; Sadun, 1963), many problems are inherent in this approach. The use of living organisms, such as sporulated oocysts, schistosome cercariae, or infective nematode larvae, as a source of antigen requires the maintenance of infected snails or other invertebrate intermediate hosts, or of infected dogs, sheep, cattle, or flocks of birds, according to the host requirements of particular parasite species.

As Lumsden (1965) has pointed out in the case of trypanosomes, the continuous syringe passage from host to host in the laboratory of the blood stages of strains of parasites that in nature normally pass through invertebrate vectors tends to result in strains that are fundamentally altered in morphology, susceptibility to drugs, infectivity to arthropod vectors, and antigenic characteristics. The situation is further complicated by the evidence that the artificial maintenance of the Tsetse fly vector of trypanosomes can have a considerable effect on the infection of this intermediate host (Weitz, 1964). However, an important advantage in using the arthropod vector hosts is that although the antigenic character of trypanosomes alters during their vertebrate host stage, when they are passed through the invertebrate host they revert to the antigenic variant of the original parent strain (Gray, 1965). The experiences with trypanosomes emphasize the need to work with cyclically transmitted parasites when this is the natural mode of infection.

Presumably, similar considerations apply to other arthropod-vectored parasites such as malaria, and from the viewpoint of providing a source of relevant and effective antigen(s) for potential vaccines, these considerations greatly complicate antigen production. This is particularly so when the need becomes apparent for large quantities of arthropods for the production of particular parasite stages (such as sporozoite antigens) for an effective malarial vaccine.

In the case of the live vaccine against fowl coccidiosis, the maintenance of numerous monospecific strains in infected birds has been successfully established for commercial production. A mixture of eight species of sporulated oocysts (*Eimeria tenella, E. necatrix, E. hagani, E. acervulina, E. maxima, E. brunetti, E. praecox, E. mivati*) is available* to be used in drinking water, and it is claimed that under controlled conditions of feed and litter management, sufficient immunity against both cecal and intestinal coccidiosis results from the initial infection and subsequent controlled reinfection to protect birds without producing undue pathologic effects. If symptoms warrant, treatment with coccidiostats such as sulfaquinoxaline or sulfamethazine are recommended.

Although insufficient evidence is available for drawing firm conclusions, there are enough indications to suggest that the physiology of the infected host has an influence on the virulence, physiology, and antigenicity of the parasite. How this effect is translated into variations of protection-stimulating antigens in infected laboratory animals is still unclear but requires early investigation.

The regular production of third-stage larvae of the lung worm of cattle, *Dictyocaulus viviparus*, using calves as donor animals, has been successfully operating since 1959 (*see* Poynter et al., 1960). The complexity of such an operation makes standardization almost impossible. However, the use of the normal host with its selective pressures in both the production of lung worm larvae and, as described above, fowl coccidial oocysts seems to ensure that an adequately antigenic organism is produced. Compensation for variations in parasites harvested from infection of any single host is provided by pooling material from a number of donor animals. Even with all of its complexities and its dependence upon the maintenance of groups of infected donor animals, this approach has proved to be amenable to veterinary commercial production methods; however, it has obvious limitations, especially when human parasites are considered.

In vitro cultivation

The *in vitro* cultivation of bacteria and viruses in cell-free media or tissue cultures has long provided the basis for antigen production for bacterial and viral vaccines. The standardization of both *in vitro* production techniques and the resultant harvested antigens has been the foundation of successful microbial vaccines.

* Coccivac®, Dorn and Mitchell Laboratories, Inc., Opelika, Alabama.

Similarly, it has become acutely apparent that cultivation techniques must be developed to maintain parasitic protozoa and metazoa *in vitro* before significant steps in the evolution of parasite vaccines can take place. The problem areas include diverse and formidable challenges such as mammalian erythrocyte and tissue culture maintenance, insect tissue culture, and cultivation of intact metazoan helminths. As great as the problems are, encouraging results have been reported for the *in vitro* maintenance of erythrocytic stages of rodent, simian, and human plasmodia (Geiman et al., 1966; Polet, 1966; Trager, 1966), for the exoerythrocytic stages of avian malaria (Huff, 1966), for the *in vitro* culture of the mosquito phase of avian malaria (Ball, 1966), for the intracellular stages of leishmania, for both the intra- and extracellular stages of trypanosomes and toxoplasma (*see* Zuckerman, 1966), and for the early intracellular stages of coccidia (Strout et al., 1965). Berntzen (1966) has made important technologic contributions toward the goal of maintaining cestode and nematode helminths *in vitro* through successive generations (*see also* Silverman, 1965b).

The need for intensive research in these areas cannot be overemphasized. The number of technologic problems to be resolved are enormous; however, the potential effect of readily available *in vitro* culture methods for parasites is immense. This would be useful not only from the immunologic aspect, but also as a tool for examining many facets of the host-parasite relationship. So far, the development of cultivation methods for parasitologic research has attracted a relatively small number of workers. It is to be hoped that extensive exploration of all types of culture techniques as they may be applied to protozoan and metazoan parasites will be increased. The problem of finding suitable tissue media for parasites may not be as difficult as anticipated. Strout et al. (1965) found, for example, that the sporozoites of the fowl coccidian, *Eimeria acervulina*, showed little requirement for specific cell species and underwent development in chicken, mouse, and human cells. Shumard and co-workers (1967, personal communication) have found that sporozoites of the fowl coccidian, *Eimeria tenella*, will develop in cultures of bovine cells. The possibility of continuous culture of the trophozoite stages of tissue and hemotropic parasites now seems an exciting feasibility. The lack of host specificity as observed in the *in vitro* cultures raises many interesting questions and suggests the use of cultured immunologically competent cells to test the role of the host response in determining host specificity.

The use of *in vitro* tissue culture methods for producing attenuated strains of parasites is another possibility and has been suggested by the work of Weiss and DeGiusti (1964, 1966). Working with a mouse strain of *P. berghei*, they found that after passage through rats and tissue culture in a medium containing lamb serum, the plasmodium became modified so that when reinoculated into susceptible mice, it stimulated sterile immunity without apparently establishing a primary patent infection. The further application of this technique to other hemotropic parasites appears warranted.

Sanders and Wallace (1966) found that trypanosomes which have been attentuated by a level of x-irradiation that prevented infection of rats with the still motile protozoa were capable of stimulating an apparent sterile

immunity. These results suggest support for the work of Thillet and Chandler (1957), who found that the metabolic products collected from *in vitro* culture fluids were effective immunizing antigens. It seems, therefore, that somatic constituents alone are not effective by themselves.

Silverman, Poynter, and Podger (1962) and, more recently, Silverman, Alger, and Hansen (1966) have used antigens produced in *in vitro* cultures by the histotropic stages of nematodes for immunization procedures. For those nematodes so far examined, chemically defined media have been utilized. The opportunity of using these model systems is worthy of more attention, since little is known of the *in vitro* culture factors which affect the quality and quantity of antigen production. The best conditions for harvesting such material and the exploration of suitable methods for concentrating and storing functional antigens need to be determined. The development of serologic and biochemical standards for assessing antigenicity is one of the most immediate challenges.

The further development of *in vitro* culture methods, preferably continuous culture procedures, will require research into many fundamental problems. These include an analysis of the factors which, in nature, trigger the intrinsic biologic clock mechanisms and signal the initiation of physiologic and morphologic changes associated with growth, development, and metamorphosis of the various parasitic stages (*see* Rogers, 1962). These factors include specific enzymes or biologic substances elaborated by the host, physicochemical conditions, gas-phase interrelationships, and temperature effects. Optimal concentration of parasites in culture media and the need to alter conditions at critical times must be determined. An attempt to simulate the conditions of vertebrate or invertebrate host environment does not always prove to be the most profitable approach. Other, sometimes surprising, culture materials have been found to be useful. Jackson (1962), for example, found that a medium similar to that designed by Trager (1957) for the leptomonad or insect-inhabiting stage of *Leishmania tarentolae* was a suitable culture environment for the nematode, *Neoaplectana glaseri*; Hansen et al. (1966) found that a medium designed for the free-living nematode, *Caenorhabditis briggsae* was suitable for the parasitic helminth, *Haemonchus contortus*.

Quality of parasite antigens

In a report (1965) of a World Health Organization Expert Committee on Immunology and Parasitic Diseases, the following recommendations were made:

> The logical and probably necessary first step toward achieving immunological measures of host resistance and immunological means of inducing or increasing that resistance is the definition of antigens related to critical stages of parasite development. Field testing with crude preparations of multiple antigens is not likely to add significantly to our knowledge of the pathogenesis of parasitic diseases If essential components of the parasite, preferably those in contact with

the host environment, can be isolated and found antigenic, they would serve as the best possible test and immunizing agents. The urgent necessity for such studies has emerged from the discussion in many places in the report and especially in the following areas:

(1) the differentiation in various parasitic diseases between
   (a) non-protective antigens (which may be useful in diagnosis) and
   (b) protective antigens (necessary in immunoprophylaxis and possibly also in diagnosis);
(2) the search for antigens common to parasites and the tissues of their particular host species;
(3) the chemical nature of the antigens secreted at natural orifices in helminths, as these may possibly be involved in protective immunity;
(4) antigenic analysis of moulting fluids of helminths;
(5) morphological and physiological studies in helminths that would lead to identification of antigenic structures, e.g., in the cuticle;
(6) the characterization of anti-enzymes, inasmuch as parasite enzymes are important in penetration and migration.

The recommendations of the W.H.O. Expert Committee allude to important qualitative aspects of parasite antigens which bear on potential vaccines. First of all, the functional antigens which appear to be important in stimulating resistance seem to be characterized by low antigenicity. The reasons for this are unknown, but speculation has centered on the enzymatic nature of these antigens. It is known that proteins which fulfill similar functions in different species may be chemically similar and this may account, at least in part, for reduced antigenicity. For this and other reasons, the need to isolate and concentrate functional antigens is a primary objective. The mosaic of antigens which are elaborated by or extracted from parasites contains a wide variety of potentially immunogenic material, and the serologic characterization of such mosaics is of little significance unless the data are closely correlated with *in vivo* protection tests. Unfortunately, very few critical studies of this type have been undertaken, but the work of Mills and Kent (1965) with *Trichinella spiralis* serves as an outstanding example of the critical studies. Another promising approach is the one used by Rhodes et al. (1965), who partially purified the enzyme, malic dehydrogenase, from *Ascaris suum* and found that it conferred some protection when injected into guinea pigs.

Secondly, the reference by the W.H.O. committee to antigens common to parasites and hosts touches on the analysis of the evolution of host-parasite relationships which affect the ability of hosts to recognize parasite antigens. Damian (1964), in an excellent review, proposed the concept of "eclipsed antigens" for an antigenic determinant of parasite origin which resembles an antigenic determinant of its host. There is considerable evidence from studies on microbes and parasites that eclipsed antigens are widespread. Obviously, if important components of potentially functional antigens become eclipsed, then the ability of a host to recognize them and to produce appropriate antibodies is lessened. Damian underlined the evolutionary forces selecting both hosts and parasite. Cogently, he proposed that one of the most effective defense mechanisms operating against the complete selection of eclipsed parasite antigens is host-antigenic polymorphism. The parasite, under this scheme, would overwhelm nonreactive hosts and select from host populations

survivors with alternative antigenic states. That such polymorphic antigenic potential exists in vertebrate hosts is evident from many examples. As Schad (1966) pointed out, where such eclipsed antigens are evident, it might be possible to enhance recognition by using antigens from closely related parasites. In Schad's review, a number of reports are summarized indicating that heterologous species may interfere with a competing parasite within the same host by an immunologic mechanism even when homologous antigenicity is low or absent.

IMMUNIZING PROCEDURES

Adjuvants and routes of inoculation

It is repeatedly emphasized in reports of successful immunization procedures that adjuvants enhance the protection-stimulating effect of the crude antigen (e.g., Silverman et al., 1962; Targett and Fulton, 1965; Richards, 1966). Freund et al. (1945), using adjuvanted dead trophozoite preparations of *P. knowlesi*, successfully immunized monkeys. However, when Heidelberger et al. (1946a, b), in a series of heroic human experiments, tried to use nonadjuvanted trophozoites of *P. vivax*, they were unable to demonstrate any immunity either to the injected erythrocyte stage of the parasite or to challenge infections by mosquito bite. The omission of adjuvant appears to have been an important factor. This has been recently illustrated by the work of Targett and Fulton (1965), who found that when they omitted adjuvant and vaccinated with antigen alone, no protection was conferred in monkeys to *P. knowlesi*.

The use of adjuvants as an accepted means of enhancing antigenicity, whether it be for the induction of resistance or the production of antisera, is testimony that the poor quality of parasitic antigens is recognized by research workers. In spite of the obvious importance of adjuvants, no critical studies on the relative merits and effects of various adjuvants have been reported.

Freund's complete adjuvant consists of a stabilized emulsion of mineral oil and mycobacteria. It has obvious limitations for human use, although its efficacy is unrivaled in enhancing antigenicity. The mineral oil component is a potential carcinogenic agent, and the injection of mycobacteria would interfere with tuberculosis tests, in addition to producing unwelcome lesions. In an attempt to find useful alternatives, Silverman (1963) compared the adjuvant effects of sodium alginate, water in oil and oil in water emulsions aluminum hydroxide, and Freund's preparation. Using nematode antigens, i was found that aluminum hydroxide, although not quite as effective a: Freund's preparation, enhanced the production of more specific antisera an stimulated minimal host tissue reactions. The adjuvant was found to b. effective in enhancing the production of resistance (Silverman et al., 1962)

Other adjuvants such as cholesterol and saponin (Richards, 1966) have been used to good advantage with parasitic antigens. The advantages of various adjuvants for specific antigens cannot be predicted, and experimental work to determine optimal combinations will have to be undertaken for each test material. The implications and importance of adjuvants for vaccines must not be underestimated.

Recently, Schelling (1966), using ovalbumin, the lipopolysaccharide of *Escherichia coli* bacteria, and somatic and metabolic antigens of the parasitic nematode, *Haemonchus contortus*, compared the effect of various routes of injection on the antibody response in rabbits. All three antigens were adjuvanted with sodium alginate, and comparable quantities were used for each injection. The intravenous, intramuscular, intradermal, and intraperitoneal routes of injection were tested for each antigen, and two injections were given within an 18-day interval. Antibody titers were measured by the tanned-cell passive hemagglutination technique, and all sera were subjected to treatment with 2-mercaptoethanol to distinguish between 19S and 7S globulins. The serum titers measured in response to the injection of ovalbumin and the *H. contortus* antigens were found to be entirely due to 7S gamma-globulin, and the intravenous and intramuscular routes consistently elicited the highest antibody titers and sustained the longest duration of antibody production. The primary antibody response to *E. coli* lipopolysaccharide was found to be 19S globulin, and the most effective route of injection proved to be the intradermal route. This work suggests that the efficacy of an injection route depends to some degree on the type of antibody elicited.

The importance of injection routes and adjuvants can be better appreciated when more information on protective antibodies is available. Cohen and McGregor (1963) showed that the protective properties of human immune serum were associated with the 7S gamma-globulin fraction. As Tobie et al. (1966) pointed out in their study on immunoglobulins in human malaria, for the induction of 7S antibody the antigen dose requirement was at least 50 times as great as that for 19S antibody. Similar studies on other parasitic infections are needed to determine both the qualitative and quantitative thresholds required for the protective immune response.

Antigen-antibody complexes

In recent years, immunologists have been investigating the role of soluble antigen-antibody complexes in stimulating an immune response. It has been found, for example, that in several types of hypersensitivity reactions the *in vitro*-formed complex is much more active than the antigen alone. Further studies have shown that such complexes form *in vivo* and are capable of initiating biologic reactions which could not be produced either by antigen or antibody alone. These phenomena were reviewed by Weigle (1961), who also summarized some of the work which suggests that the complexes may be operating either as a stable unit or that they may be effecting their activity

by a process of dissociation and reassociation. The mechanism of action is not clearly understood; however, it is obvious that such complexes are formed in nature and that preformed *in vitro* complexes (in the proper proportion of antigen to antibody) result in a greatly enhanced antigenicity. The use of preformed *in vitro* antigen-antibody complexes as a natural adjuvanting procedure for parasite antigens is strongly suggested.

Stumberg (1933), after the demonstration by Stoll (1929) that sheep acquire resistance to *Haemonchus contortus*, examined the sera of resistant and infected animals. By means of an anaphylactic test in guinea pigs, he found evidence which strongly suggested that *H. contortus* antigen occurred in the serum of sheep. The possible role of soluble antigen-antibody complexes was the subject of recent studies by Ristic (1966) and his co-workers on *Babesia* and *Plasmodium*. They have found serum-soluble antigens which can be used for the diagnosis of the disease and which, when used as immunizing agents, confer protection on dogs challenged with babesiosis (Sibinovic, 1966) or chickens challenged with malaria (Todorovic et al., 1966). A possible explanation for the presence of antigens in the serum is that they represent antigen-antibody complexes consisting of parasite metabolites and host antibody. These are exciting results which ought to be further pursued with vigor, and they suggest similar lines of inquiry for other parasitic infections.

Quantitative factors

The existence of immunologic thresholds in animals has long been established in the field of bacteriology (*see* Wilson and Miles, 1964). Below a certain minimum quantity of whole bacilli or bacterial polysaccharide or protein antigen, no immunologic response is induced. During the induction period of the primary response, it appears necessary to maintain a minimum level of circulating antigen in the serum. The threshold necessary must be maintained either by a sufficiently large single inoculation or by a series of multiple injections. On the other hand, too much antigen can induce unresponsiveness in the form of immunologic paralysis. Immunologic paralysis inhibits an animal from responding to an antigen for varying lengths of times (i.e., from weeks to months). However, following recovery from immunologic paralysis, the animal can respond normally to the original immunologically paralyzing antigen if stimulated at an appropriate threshold level.

In a critical study of quantitative factors affecting the primary response Mitchison (1964), using bovine serum albumin as an antigen in mice, found two dosage zones, one low and one high, at which immunologic paralysis rather than antibody production was induced. He also found dramatic differences when adjuvant was used, when multiple injections were given, or when the interval between injections was reduced. With respect to the secondary complex, additional observations by Mitchison (1964) and Makela and Mitchison (1965) may explain the puzzling reports that prior immunization with

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parasite antigens exacerbated the challenge infection over that seen in the controls. Studying the quantitative effects of bovine and human serum albumin on the ability of primed cells to respond to a second antigenic stimulus and using adoptively transferred cells in syngenic hosts, they found that as little as 10^{-3} μg of BSA was needed to stimulate a secondary response. At higher dose ranges (10^{5} μg), the proliferation of immunocytes and synthesis of antibody were inhibited. The significance of these results in terms of attempts to immunize artificially against parasites is obvious. Tests with potential functional antigens must be planned over a range of dosage levels and using varying schedules.

In addition, a determination of the most appropriate size of challenge dose and how it is to be administered must be considered. Little definitive information is available on the rate and number of infective stages which host animals are apt to be exposed to in nature. Thus, a single massive dose of the infective stage of a parasite presented to an experimentally immunized animal might completely overwhelm the immune response and suggest that no immunity has been established. On the other hand, in nature, the animal may never encounter more than a small number of infective stages presented at intervals in the form of a "trickle" challenge. Indeed, much more information is needed, e.g., on the number of sporozoites, infective nematode larvae, or trematode cercariae, to which hosts are exposed under normal epidemiologic conditions.

The effects of small quantities of "good" antigens on the type of immune response have been studied by Salvin and Smith (1959, 1960). Using a variety of protein antigens and purified diphtheria toxoid, they found that in guinea pigs after injection with very low doses of antigen, a delayed hypersensitivity reaction was observed but no circulating antibody was detected. Further stimulation of the sensitized guinea pig with the specific homologous antigen resulted in an anamnestic response during which both circulating antibody and Arthus-type hypersensitivity developed. These data support the suggestion that delayed hypersensitivity is an intermediate stage in the formation of circulating antibody. The delayed hypersensitivity reaction has often been reported as a result of infection with parasites or inoculation with parasite antigens. This would tend to suggest that in terms of the model proposed by Salvin and Smith, the host animal was only partially immunized, and that further stimulation would be necessary for a complete circulating antibody response. During natural infection, the inadequacy of antigenic stimulation for the production of circulating antibody because of qualitative and quantitative reasons may explain why resistance is slow in developing and why serologic tests yield low titers. The transitory nature of functional antigens contributes to the difficulty in obtaining a minimum immunologic threshold. Conceivably, the number of parasites required to produce sufficient antigen during the induction phase may be of such an order that the concurrent pathologic stress adversely affects antibody synthesis by the host.

STANDARDIZATION

Criteria

The aim of vaccination against microbial infectious diseases is to prevent disease and mortality. The fact that populations immunized against polio continue to excrete virulent viruses or that clostridial spores can be isolated from symptomless hosts is accepted by microbiologists as a sign of successful vaccination. Reduction in morbidity and mortality are the main criteria used in assessing the potency of vaccines against poliomyelitis, diphtheria, tetanus, pertussis, erysipelas, cholera, pneumonia, smallpox, and rabies. It is not necessary to demonstrate that the protected host is completely free of the infectious agent, i.e., that sterile immunity exists.

Unfortunately, the same criteria have not always been applicable to studies on parasite vaccines. In many protozoan and metazoan parasitic infections, the disease tends to be chronic in nature rather than acute, so the lethal end-points are difficult to establish. A wide variety of criteria are described in the preceding chapters, and most of them deal with reduction in degree of parasitization instead of mortality. The use of reduced parasitization as an index of partial or complete immunity is fraught with many problems, and more subtle criteria for assessing different levels of host morbidity and parasite inhibition must be established. Furthermore, on the basis of data related to dosage loads in nature (referred to earlier), a more reasonable assessment of the levels of immunity to be attained can be determined.

The expectation that vaccination will lead to parasite-free individuals is unrealistic on the basis of present evidence. In fact, in certain epidemiologic situations, this might be unwise. It appears that the maintenance of a very low level of nonpathogenic parasitization may be desirable, since this may help to maintain an adequate level of resistance by providing a constant source of antigenic stimulation. The aim at this stage appears to be best illustrated by the case of adults in hyperendemic malarious areas who have developed resistance and show very few parasites in their circulating erythrocytes. When such persons are removed from their native environments for long periods of time and then return, they suffer a fresh but mild malaria attack. Similarly, domestic animals which develop resistance to helminth infections during the summer and autumn apparently lose some of this immunity during the winter when transmission of infective larvae is at a low ebb. As a result, the animals become reinfected with low levels of parasites in the spring, but quickly regain their previous state of resistance.

Potency and safety

The establishment of potency standards for widely used vaccines has occupied microbiologists for many years. A wide range of techniques and stand-

ards has been used and is accepted on an international basis (*see* World Health Organization Tech. Rep. Ser. No. 222, 1961). In general, potency is measured by comparison of an unknown vaccine preparation with a known antigen or standard antiserum in a laboratory-animal protection test. Potency testing may use natural routes or highly artificial routes of challenge, i.e., intraperitoneal or intracerebral inoculation. The important factor is that the test measures the same kind of immunity that it is desired to induce. The actual units are arbitrary, but some reference standard must always be available. The range for effective vaccine doses can be fairly wide but must provide some margin of error so that effective doses do not become ineffective because of too little stimulation or result in immunologic paralysis because of too much antigen.

Several widely used microbial vaccines have not yet been standardized because they depend on living organisms. With biologic products such as yellow fever, smallpox, and B.C.G. vaccines, direct tests on animals in order to indicate potency have to be carried out. It is perhaps needless to point out that in all cases the ability to store vaccine material until potency and safety factors have been established is a prerequisite. So-called shelf-life studies become important in providing for an adequate period of potency and safety testing and in allowing for distribution to the user.

The question of safety standards becomes particularly important when living organisms are used for vaccination. In the case of attenuation by passage through abnormal hosts, by irradiation, or by *in vitro* cultivation, each batch must be tested to ensure that attenuation has been effected so that potential pathogens can be eliminated. In the case of certain organisms such as infective nematode larvae that are reared in highly contaminated fecal media, it is necessary to ensure that the cleansing process has removed the potentially infective microbes that might be present in association with the larvae. In this regard, the problem is further complicated by the fact that bacteria and viruses can survive in the intestine of the nematode even after extensive cleansing of the cuticular surfaces.

For reasons of potency and safety standardization and to provide for maximal shelf life, dead vaccines offer many advantages over live ones. Undoubtedly, the process of research to produce dead vaccines by isolating and enhancing the activity of the functional antigens is a long and tedious one. Nevertheless, though there is much justification for the use of living vaccines, the attempt to understand the immune mechanisms and to produce standardized immunologic products must continue.

CONCLUSION

One of the striking impressions gained from a review of the literature in the fields of microbial and parasitic immunity and vaccination is the similarity of problems. These common problems are found at every level of study of host-parasite relations, infection and transmission, *in vitro* cultivation, sero-

logic evaluation, antigen preparations, immunization, and parasite selection and evolution, to name only a few areas of obvious common interest. In this regard, bacteriologists, virologists, and parasitologists concerned with immune mechanisms and prophylactic immunization have much to learn from one another. Also, the theoretically inclined immunologist working with model systems and artificial antigens provides a source of new insight and ideas for experimentation with the crude parasite antigens so far available.

Immunoparasitologists have too often in the past focused narrowly on single organisms or on a limited group of parasites. Papers published on the immune response of one species of parasite often refer to literature related only to that species and ignore relevant work done on other even closely related organisms. As a result, a mystique of parasite "exceptionalisms" has developed with the implication that each host-parasite relationship is unique.

Quantitative phenomena related to antigenic stimulation and the development of resistance cannot always be conveniently studied with all parasites, and guidelines must be sought from whatever source available. Similarly, information on the effects of adjuvants, routes of inoculation, immunization schedules, and the qualitative alterations to antigens which lead to an enhanced host immune response or immune paralysis are of interest, no matter what the source of antigen used for such studies may be.

Basic to the approach outlined in this chapter are the convictions that a host immune response is a normal reaction to parasitism and that wherever such immune responses have protective value, it should be possible to stimulate immunity artificially. The opportunity to review the literature afforded by the occasion of writing this chapter has further deepened this conviction.

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