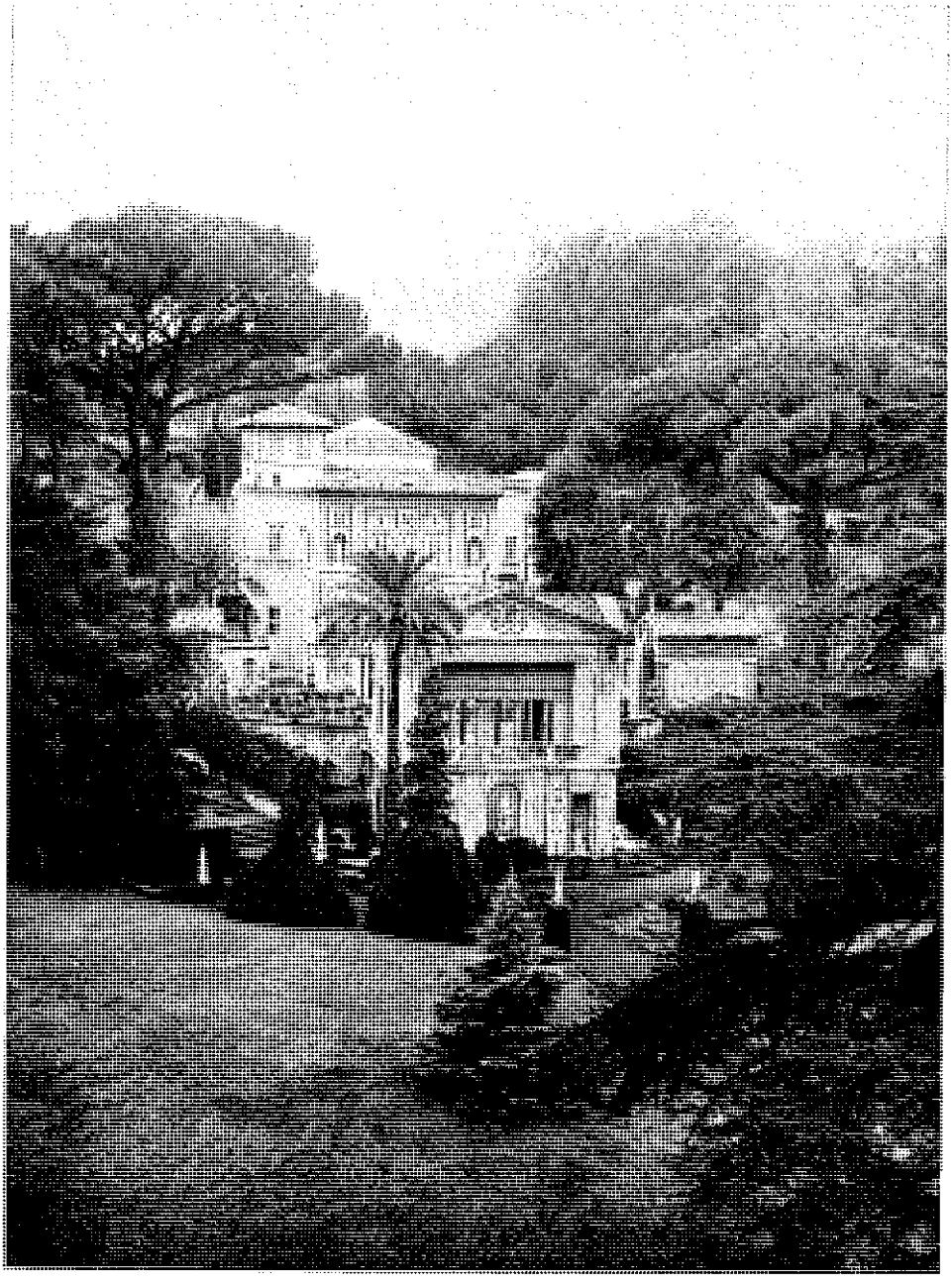

PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA

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WORKING GROUP

ON:

PERSPECTIVES OF IMMUNIZATION IN PARASITIC DISEASES

September 29 - October 2, 1981

EDITED BY

CARLOS CHAGAS

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FOREWORD

The explosive developments occurring in the field of immunology have opened new perspectives for the production of vaccines against some of the worst diseases which affect humanity.

The concept of immunization, which was, I believe, first perceived by the Greek philosopher Thucydides — when he described an epidemic which occurred in Athens, circa 500 B.C., probably produced by plague — gained attention with the work of Jenner towards the end of the XVIIIth century. It acquired its scientific background as a result of Pasteur's and Metchnikoff's work, undertaken in the last quarter of the XIXth century.

In the field of parasitic diseases humanity faces a great challenge as it is believed that close to a billion individuals are prey to these diseases.

Endemic regions around the world are the sites of extensive research, sponsored by governmental health agencies. This research is not only directed towards the control or eradication of the disease itself, but involves also a complete study of the sanitary conditions of the population, which in most cases provide a "healthy" habitat for the parasite's vector (e.g., the case of Chagas' disease, where the vector inhabits the thatch roofs of rural huts).

Therefore, parasitic diseases can be fought by prevention, which necessarily includes the improvement of socio-economic conditions, by therapy where it exists, and by education. Prevention and education, including the elimination of the parasite's vector, is in the foreground of modern medical directives as it seems wiser to provide health for humanity, thus rendering therapy less necessary.

It becomes each day clearer that immunization, or vaccination to employ a more usual term, is a powerful weapon for preventive medicine. To prove its success it is enough to point out the recent eradication of smallpox throughout the world, the control of yellow fever and poliomyelitis. Modern immunology, with all the support it receives from molecular biology, biochemistry and genetics, is therefore a promising tool for fighting some of the challenges modern society faces.

It seems, therefore, not too idealistic to approach through immunization the problem of prevention of diseases whose pathogenic agents are parasites.

In some cases this approach is quite compulsory. Some vectors have become or are becoming resistant to the known pesticides and may develop resistance to new products; in turn, the parasites become resistant to the drugs used in therapy. Such is, for instance, the case of malaria.

It was with these facts in mind that the Pontifical Academy of Sciences held a meeting of specialists at its seat in the Vatican to try to form an overall picture of the perspectives of immunization in five parasitic diseases: malaria, schistosomiasis, leishmaniasis, African and American trypanosomiasis. As our resources were limited, a choice had to be made. The diseases chosen were those on which more immunological work has been done, bringing the perspectives of vaccination closer, or where the perspectives seem still very remote, as in the case of American trypanosomiasis.

I hope that the results published in this volume will be of use to the STD program in which the UNDP/World Bank/WHO are directly involved, in the study, control and eradication of "Tropical" diseases. The strenuous task accomplished by the participants of the Working Group will also be useful for governments in shaping their health and biomedical research policies. This publication may also interest researchers around the world.

I wish to thank the participants of the Working Group who so promptly accepted our invitation and labored for five long days in the Casina Pio IV. The results which will no doubt be achieved, are due to the zeal and scientific knowledge which they brought to the meeting. The Working Group was chaired by Pontifical Academician Prof. Percy Garnham, whose wisdom and experience were invaluable to the success of our endeavor.

Our gratitude is extended to Father di Rovasenda, Director of the Chancery of the Academy; Mrs. Porcelli-Studer, secretary of the Academy, and Mr. Silvio Devoto. Without their interest and devotion it would not have been possible to hold the meeting.

Let us hope our objectives will be attained.

Rome, October 1981

Prof. CARLOS CHAGAS
President of the
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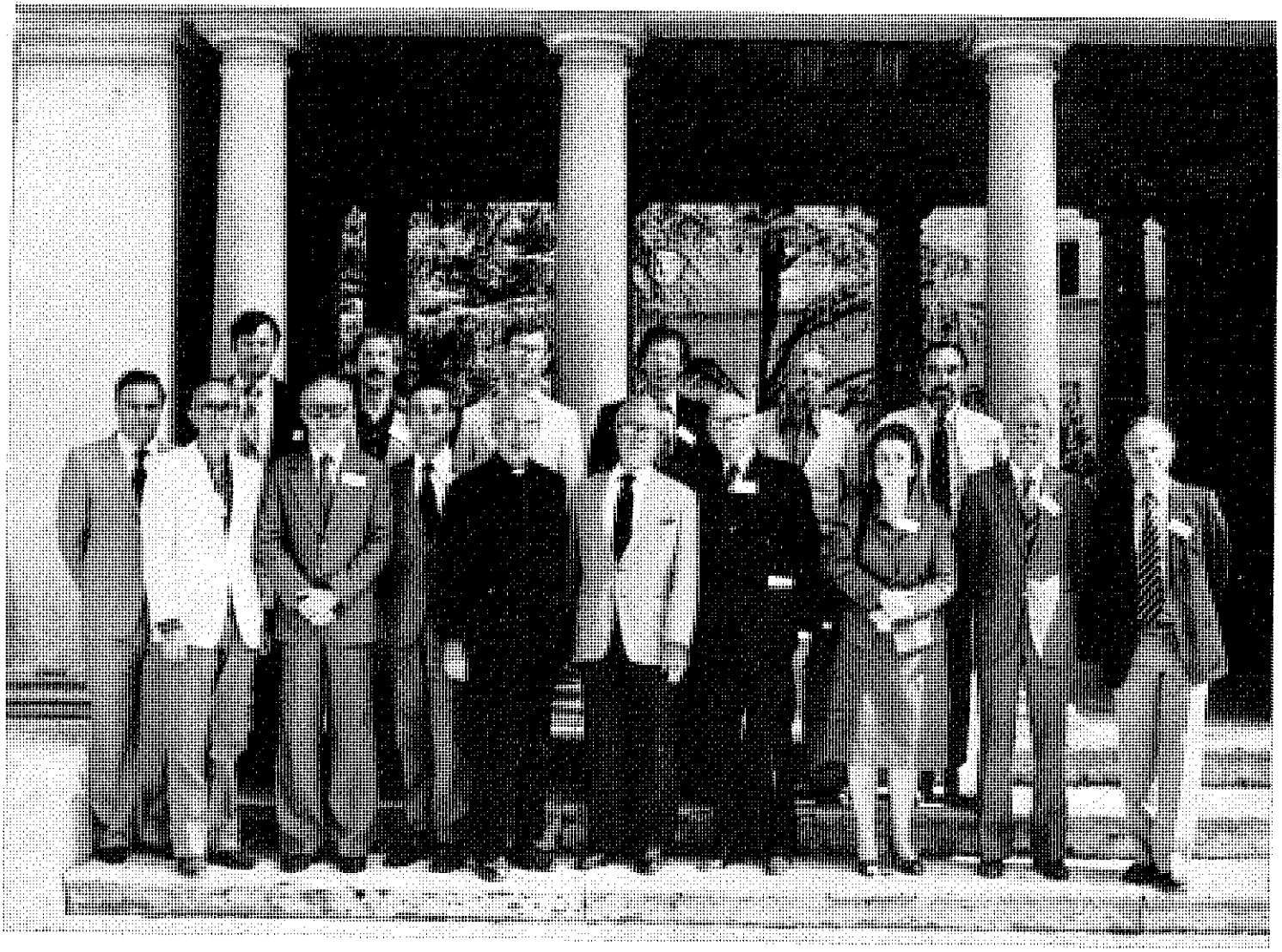

SCIENTIFIC PAPERS

INTRODUCTORY REMARKS TO PERSPECTIVES OF IMMUNIZATION IN PARASITIC DISEASES

P. C.C. GARNHAM

I am glad to have the opportunity of meeting, in the Pontifical Academy of Sciences, many of my old friends in the parasitology field. I hope that during the coming week we shall be able to examine in some detail the popular and important subject of vaccination in certain parasitic diseases — African and American Trypanosomiasis, Leishmaniasis, Malaria and Schistosomiasis — which affect the Third World and cause enormous morbidity and mortality. There are of course other diseases of importance, but these 5 comprise the major part of the « Special Programme » of W.H.O.

Like many of you, I have attended numerous conferences on parasitic immunity, and nearly 20 years ago, Dr. Joyner, Dr. Roitt and I organized the First International Meeting on Immunity in the Protozoa (published under this title in 1963); the latest one was on « Vaccines against Parasites » (1980) — a Symposium of the British Society for Parasitology. The former largely represented the thoughts of the old school, the latter was typical of the modern approach, which even now is becoming dated — as illustrated by the advances described in your own manuscripts.

Two new avenues have opened for the control or eradication of parasitic infections: 1. *Biological control*, still in its infancy, but showing increasing promise, e.g., the discovery of a new subspecies of *Bacillus thuringiensis* in Israel [by Margalit (*Biologist* 1981, *28*, 194)], which is vigorously toxic to mosquito larva. 2. *Vaccination*, hardly a « new » weapon, as it has been used for centuries in the Middle East against

leishmaniasis and highly successfully in recent years in the USSR, while the use of Jenner's cowpox virus dates from nearly 200 years ago. But there has been a sudden burst of activity in the last decade on vaccines, based on molecular biology and a new look at the nature of immunity.

The necessity for a new approach has been highlighted by the increasing incidence of resistance of parasites to drugs and of arthropods to pesticides.

In the course of our deliberations, I hope that we shall bear in mind certain general considerations other than the technical aspects of immunity. It is useful to consider the three following possibilities:

1) Is a vector — snail, arthropod etc. — involved in the life cycle of the parasite?

2) Is there an obligatory vertebrate host (e.g. a cow) concerned in the life cycle of the parasite (e.g. Taenia saginata)?

3) Is there an animal reservoir?

Vaccination may be highly successful if the answers to these questions are negative. If the answers are positive, the situation becomes more complex; thus if an animal reservoir lurks in the background, as in certain forms of leishmaniasis (e.g. due to L. mexicanum in arboreal rodents), Chagas' disease (with opposums and other wild animals as reservoirs), or yellow fever (with the huge population of monkeys infected with the virus in Africa and Latin America) eradication (though not control) will be practically impossible.

A very important practical consideration is the accessibility of the population to vaccination. There are at least two points:

1) Is the population sufficiently organised and educated to allow of a steady and complete progress of vaccination? Although smallpox vaccination has been completely successful, it is doubtful if more sophisticated procedures would be acceptable and could be carried out in the less advanced regions of the world.

2) The age structure of the population is an important factor as Dr. Mott will show in his paper in special relation to malaria, and as R.M. Anderson and R.M. May have indicated in a paper (« The Epidemiology of Directly Transmitted Infectious Diseases: Control by Vaccination » to be published in *Science*) in which it is stated that this factor may operate adversely in malaria control.

Although this meeting is to consider only these five human diseases, prospects exist for the control of some other infections by vaccination. This especially applies to certain veterinary problems in the Third World, e.g. the introduction of a lungworm vaccine which has been in routine use for over 21 years, the combined attack by a vaccine made from attenuated living trypanosomes (*T. Congolense*) and small doses of drugs, and the use of vaccines of *Theileria annulata* rendered non-pathogenic after continuous tissue culture of the schizonts. Nobody as yet appears to have attempted to introduce a vaccine for the prophylaxis of the widespread and serious disease — amoebiasis, and in certain circumstances such a vaccine would be invaluable.

The ultimate aim of a vaccine must be the introduction of a protective antigen in a chemically identifiable form and capable of synthetic production. When I suggested this idea to Prof. Stanley Cohen, he said that we would have to wait for many years before such materials were synthesised. Today, however, the production of monoclonal antibodies and the incorporation of the protective gene into bacteria and phage appear to offer the possibility of by-passing this chemical approach. I believe that we shall hear something of such methods during the course of this meeting.

EPIDEMIOLOGICAL CONSIDERATIONS FOR PARASITE VACCINE DEVELOPMENT

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Parasitism or for that matter polyparasitism is almost a ubiquitous phenomenon in the endemic areas so that it is worthwhile to make certain that we observe the behavior of parasites in the human populations.

Epidemiological observations on parasitic infections may provide information on:

1) The rate of acquisition of natural infection.

2) The interrelationship between prevalence and intensity of infection in relation to known characteristics of transmission.

3) The presence of naturally acquired immunity in affected populations.

4) Expected status of parasitic infection in different age and sex groups of affected population which potentially would receive a specific vaccine.

5) An indication of the factors which should be measured in evaluation of the efficacy as parasitic vaccine.

In this age of specialization it is not surprising that the comparison of the distribution of prevalence and intensity of infection of different parasitic infections in different populations let alone the same population in endemic areas has not been done. I will compare available epidemiological data from major parasitic diseases; two blood protozoal infections, malaria and Chagas' disease and schistosomiasis and filariasis and discuss the possible interpretations of these data in relation to the acquisition of natural immunity and the implications for vaccine development.

Prevalence and intensity of infection

Malaria in endemic areas has been characterized as either holoendemic or hyperendemic, mesoendemic or hypoendemic, in descending order of intensity and stability of transmission.

In hyperendemic areas by the age of one year most children are infected. At ages 1-4 years the peak intensity (highest proportion of the fields have at least one parasitized cell) of infection occurs. Although prevalence may remain high in the older age groups, the intensity of infection gradually diminishes (Graph I).

In the mesoendemic areas, by definition the prevalence is lower and the shape of the distribution curve of prevalence may vary, but in contrast to that observed in the hyperendemic area, the slope of the curve of the intensity of infection is not so great.

In the hypoendemic area, prevalence distribution is proportionally lower than in the other two situations; however, the intensity of infection remains high in all age groups. Malaria transmission in this situation is unstable and the level of prevalence may vary considerably over time (Graph II).

The undisputed natural immunity to malaria led malariologists of the past to suggest variously that a child should not be treated during his first attack of malaria or that in areas of holoendemic malaria treatment should not be given which would disrupt the established immunity in the population since the clinical severity of malaria and mortality in older children and adults would increase [39].

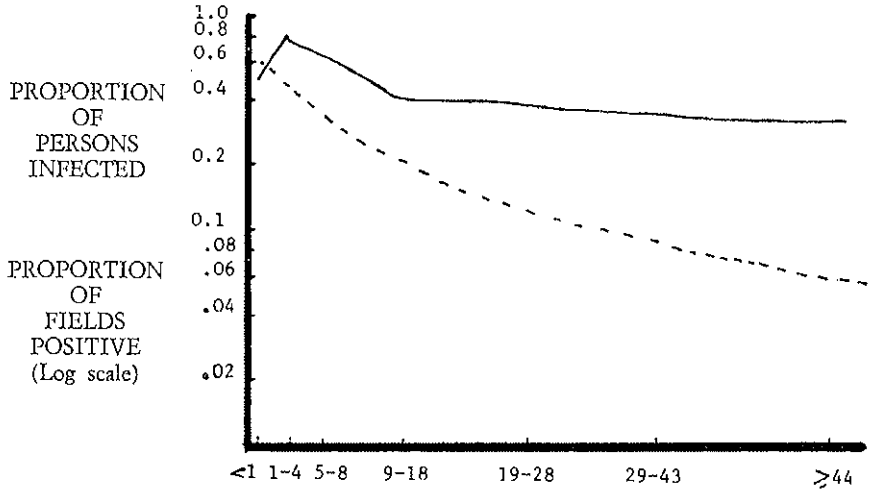
The data from the Garki project [39] in a holoendemic area indicate that the immunity related to malaria is manifested by a rapid recovery or reduction in parasitemia after infection but in fact susceptibility is unchanged. This conclusion is substantiated by the following:

1) In the dry season — when transmission is low, the net proportion of positive persons becoming negative *increases* by a factor of 10 while in the rainy season the proportion of negative becoming positive decreases with age only by a factor of 2.

2) The trophozoite density decreases faster by age than the parasite rate.

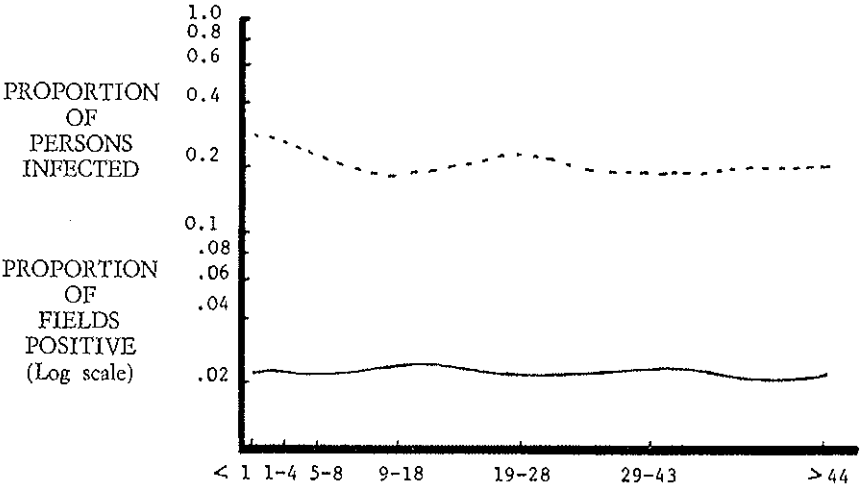
3) Both gametocyte rate and density decrease faster by age than parasite rate and trophozoite density.

MALARIA
HIGH LEVEL STABLE TRANSMISSION



Graph I

MALARIA
LOW LEVEL UNSTABLE TRANSMISSION



Graph II

4) In the age groups below age 5 the majority of those infected have high density infections. Between 5-18 the distribution of different levels of infection are similar and above age 19 very few persons are heavily infected. This pattern was similar in the wet and dry seasons.

The age specific distribution of the prevalence of *Schistosoma mansoni* infections is remarkably similar in most endemic areas [4, 26, 34, 41]. Peak prevalence is reached in the 10-25 year age groups and peak intensity of infection as measured by fecal egg counts usually is reached prior to or concomitant with peak prevalence. The prevalence rates remain constant though at a lower level after peak prevalence has been attained. The intensity of infection gradually falls to a proportion 10-15 times less than the peak prevalence of infection.

Rather than wade into the muddy waters of immunity vs epidemiology in attempting to interpret this distribution of the prevalence and intensity of infection as an isolated phenomenon, it is worthwhile to compare these distributions of different species of *Schistosoma* in human and animal populations.

Unlike *S. mansoni* infection, the peak prevalence and intensity of *S. haemotobium* infection usually occur in the same age groups — about 10-14 years of age [8, 18, 36, 56, 57]. The peak is followed by steep slope and decline in both prevalence and intensity of infection by age 30 or earlier. Obviously exceptions to this generalization can be cited but these are explained by unusual features of transmission or cultural patterns.

Antigenically *S. haematobium* is quite similar to *S. bovis* [48]. The distribution of prevalence and intensity of *S. bovis* in cattle is strikingly similar to *S. haematobium* in man i.e. 90% prevalence and peak intensity of infection occurs in three year old cattle and by 10 years, the prevalence is 10% or less and intensity of infection is low [29].

The hypothesis tbat the absence of *S. haematobium* in the Mediterranean area was due to cross reactivity to *S. bovis* led Hsu *et al.* [28] to examine the acquired resistance to challenge infection with *S. haematobium* by exposure to *S. bovis* cerceriae. In spite of this promising initial work and supporting clinical studies [19], suhsequently most efforts have been directed to understanding immunity against *S. mansoni* and development of *S. bovis* vaccine for veterinary use which currently is undergoing field testing.

Epidemiological data support the presence of natural immunity to *S. haematobium* infection more than to *S. mansoni* infection and are sug-

gestive that development of a vaccine would be more feasible against *S. haematobium* than *S. mansoni.*

Several recent population based epidemiological studies on *S. japonicum* using quantitative methods have been completed [16, 54]. The bimodal distribution of prevalence as well as intensity of infection indicates that the human immune response to *S. japonicum* may be quite different from either *S. haematobium* or *S. mansoni.*

The distribution of prevalence and intensity of nocturnally periodic *Wuchereria bancrofti* infection according to age is a remarkable contrast to the distribution patterns of malaria [p, 20, 21, 46].

The highest prevalence of *W. bancrofti* and *B. malayi* is attained after age 25 and is sustained at the same level through the older age groups. The peak intensity of microfilaremia occurs in the same age groups as the peak prevalence. Although microfilaremia may not be an accurate assessment of the number of adult worms in the human host, nevertheless it is our only means of quantitating infection.

Increasing prevalence and density of microfilaremia with age have been reported in *Brugia timori* infections as well [14, 43]. In the South Pacific form of *W. bancrofti* which is diurnally sub-periodic, this distribution has not been observed [25].

This distribution of microfilaremia in *W. bancrofti* suggests that the infection is acquired at a relatively constant rate up to about age 45 at which time the human host is refractory to new infection or eliminates the old infection.

The age specific distribution of the prevalence of parasitemia in *Chagas'* disease or American trypanosomiasis is (similar to malaria) highest in the 1-4 year old age group and gradually decreases in the older age groups [27].

Detectable parasitemia [27] and the highest levels antibody titers [40] are found more frequent in the age groups below age 20. This indicates that the host immune response is at least partially effective in reducing parasitemia.

The age specific distribution of the prevalence of antibodies to *T. cruzi* shows a gradual increase until age 20 and their similar prevalence through age 55 and lower prevalence rates in the older age groups. The level of antibody response to *T. cruzi* infection remains relatively constant after age 20 [40]. The geometric mean titer of CF and IFAT antibodies to *T. cruzi* were similar in individuals with and without detectable *T. cruzi* parasitemia.

The striking difference between the prevalence rates of seroreactivity after 50 years of age in two community studies in Brazil [40] and Venezuela [44] has puzzled us for five years. It is now apparent that the difference is due to the fact that in the Venezuela study individuals with low titer CF antibody to *T. cruzi* were considered to be seroreactive whereas in our study a titer of 1:8 or greater was the criteria for seropositivity. Furthermore, most persons with titers less than 1:8 were over 50 years of age in Brazil. In collaboration with Professor Charles Santos Buch at Cornell University Medical College sera from individuals over age 40 with essential hypertension and coronary artery disease were tested. Most of these individuals had low titer specific *T. cruzi* antibody as well as antibody to different cardiovascular cellular components. The cross reactivity between *T. cruzi* antigens and human myocardial cells must be considered in the development of a specific vaccine and in the interpretation of serological data.

Of the diseases examined, only in filariasis does the parasite density show a positive correlation with age (Table 1). This striking difference may indicate that in contrast to malaria, Schistosomiasis and Chagas' disease, the immense response to the existing parasite is altered by constant reinfection and permits gradual accumulation of parasite load.

TABLE 1 - *Proportional change of Intensity of Infection between peak infected age group and oldest age group.*

	Proportion	Age groups (years)
Malaria (High)	$-7\times$	< 1 to $\geqslant 45$
(Low)	same	
Schistosomiasis mansoni	$-9\times$	10-14 to $\geqslant 45$
haematobium	$-9\times$	10-14 to 25-29
japonicum	bimodal distribution	
Filariasis	$+10\times$	peak between 25-45
Chagas	$-1.5\times$	5-9 to $\geqslant 45$

Exposure to risk of infection

In the endemic area, the exposure to the infective parasite is a highly repetitive experience for the entire population.

In the Garki area where malaria transmission is high it was estimated that each person in the area, according to different villages received between 10-132 sporozoite inoculations per year. It might be expected that in the villages with high rates of sporozoite inoculation some evidence of decreased susceptibility could be observed, however none was observed [39].

In contrast to malaria the exposure to infective stage of *filaria* may be extremely high. The number of bites per person by *Culex fatigans* in endemic areas may be as high as 115.000 per year and such individuals would be exposed to as many as 5904 third stage infective microfilaria [21].

In schistosomiasis endemic areas quantitative measurements or estimates of the number of cerceriae penetrating the skin have not been made. Cercerial dermatitis associated with *S. japonicum* infection, particularly in non-immune persons, has rarely been reported from *S. mansoni* endemic areas. Quantitative measurements of exposure to infective trypomastigotes of *T. cruzi* are not feasible, but repeated reinfection would be expected in endemic areas.

Duration of infection

In individuals who have left the endemic area, the longevity of *Schistosoma mansoni* [52], *Schistosoma japonicum* [24] and subperiodic *Wuchereria bancrofti* [12] is known to be up to 40 years. However, within the endemic area the reproductivity, if the not viability of these parasites is much shorter [53].

Reinfection after specific treatment

Information on the immune status of treated persons in endemic areas is spare. On the Garki project mass drug administration resulted in a subsequent higher prevalence and intensity of *P. falciparum* in the

same population. However, during the resurgence the difference between parasitemia in females and males was even greater [39].

In schistosomiasis due to *S. mansoni* recent longitudinal studies have indicated that the duration of resistance to reinfection may be in the range of 2-3 years [32]. Antibody to adult *S. mansoni* antigens persists longer after treatment than antibody to *S. mansoni* eggs. However, its role in resistance to reinfection is not known [55]. Clearly more longitudinal studies are needed to clarify the immune status after specific treatment of parasitic infection.

Exposure in the perinatal period and infancy

Congenital malaria is rarely observed in high prevalence area infants born of infected mothers [13]. Serum from cord blood of infants born to naturally infected mothers can passively reduce parasitemia in older infected children [17] and newborn infants are usually not susceptible to malaria for the first 2-3 months of life. After this period high parasite density and a high case fatality rate is common. No investigations on the possible transplacental passage of malaria antigens nor the status of the cellular immune response in newborn infants have been reported.

Circulating *S. mansoni* soluble antigens (CSA) [11] as well as specific antibodies [33] have been shown to be present in fetal umbilical cord blood from placenta of infected mothers. A high proportion of newborn infants born to infected mothers in an endemic area have a delayed type of hypersensitivity to *S. mansoni* andult antigen [10]. The effect of the sensitization in relation to subsequent resistance or susceptibility to *S. mansoni* infection is not known.

It is remarkable that in highly endemic areas of schistosomiasis, acute clinically apparent disease is rarely, if ever observed. In fact the only cases of acute disease observed in an endemic area have been in individuals who have been successfully treated, in persons who have undergone complete removal of viable worms by portal filtration or in young urban residents who have never been exposed previously. This is even more striking when it is considered that in most endemic areas initial infection does not become patent until 3-4 years of age. From the epidemiological data available it seems that *S. mansoni* infection may be acquired earlier than *S. haematobium*.

In Chagas' disease we have observed that children whose mothers

were seroreactive to *T. cruzi* antigen were significantly younger than other children [40]. The conversion to seroreactivity early in life was not due solely to exposure and indicates that the immune response of these children was altered. In this same population it was found that all individuals with ECG conduction defects below age 20 had seropositive mothers. However, the small group size did not permit statistical analysis.

In established endemic areas, most clinically detected acute infections with *T. cruzi* occur below age 10 [40]. However, in an area where transmission was recently established acute Chagas' disease was observed in older persons [5].

All these previous observations support the view that in the endemic area, the autochthonous newborn infant probably does not respond to the initial infection as if it were a simple primary infection. If a vaccine is to be applied in endemic areas, even young children may have already developed a specific immune response which will altet their response to the vaccine. The immune status of the newborn infant in relation to all these parasitic diseases requires further investigation.

Sex differences in immune response

Sex differences in the humoral immune response have been noted in several bacterial and viral infections [3, 45]. In malaria such data have been analyzed infrequently in whole populations. In the Garki project [39] women had a higher total level of I gM antibody and a higher mean titer of antihodies to *P. falciparum* before and after intervention. After 5 years of age females had a lower *P. falciparum* and *P. malariae* parasite rate than males. These findings were interpreted to indicate a higher quantitative level of immunity in females. Since the parasite rate below age 5 was similar in males and females, this higher level of immunity was possibly due to a stronger immune response rather than a difference in antigenic stimulus. Furthermore in previously treated areas where malaria recurred the difference in parasite rates between males and females was greater than observed in either the baseline or control populations but unaccompanied by any change in the difference of serological titers. It was concluded that females not only had a stronger humoral response but a stronger natural immunity and/or a stronger cellular immune response. This latter observation is supported by the lack of correlation to HLA matching in survival of renal transplants

in females compared to males [42], which may be due to sex differences in cellular immunity [15].

Along another line, the antibody titer to antiviral capsid antigen (VCA) of Epstein-Barr virus have been shown to be higher in females than males in malarious area. The difference was significant as to suggest that different standards for risk assessment of Burkitt's lymphoma by measurement of VCA antibody titer should be developed for women. In the same study there was no difference in the antimalarial antibody titer according to sex [7]. No sex difference in clinical severity of malaria has been noted except in women during pregnancy [39].

In most studies on nocturnally periodic *Wuchereria bancrofti*, the prevalence and density of microfilaremia was higher in males than in females. In a survey of over 700.000 persons from Sri Lanka, the prevalence rate was similar in males and females until age 15, then the prevalence rate in females was significantly lower in subsequent age groups [2].

In areas of subperiodic *Wuchereria bancrofti* if there was not a statistically significant difference between sex-specific prevalence rates, the trend was the same [30].

In most endemic areas of filariasis the prevalence of disease related to filarial infection has been higher in males than females. However, it is possible that these observations are biased by incomplete examination of females or inability to detect lymphatic involvement in the genital area.

Females over 10 years of age had a generally higher titer of specific CF antibody to *T. cruzi* than males [40]. Although prevalence of seroreactivity was similar in both groups, the prevalence of ECG abnormalities in females was significantly lower [35].

In schistosomiasis wide cultural differences and water contact patterns provide a sound basis for explaining the observed sex differences in prevalence and intensity of infection, except in *S. japonicum* infection in the Philippines where high intensity infections were observed in older females [16, 54].

Sex differences in the development of disease which may be related to immunopathology and immune response has been suggested in relation to liver disease [49] and heart disease [45]. However, as noted in Chagas' disease and filariasis higher rates of disease in males has been observed. Sex differences in these parasitic infections are such that it would not surprise me if a sex factor affinity site such as is found on *E. coli* were found on parasite surface [23].

CLUSTERING OF INFECTION AND GENETICS

With the advent of HLA typing there has been a tendency in some quarters to rush to the field without consideration for epidemiological evidence of a possible genetic association of 1) susceptibility to infection; 2) acquisition of bigh level infection, and ultimately 3) a predisposition to development of disease — all of which will influence the evaluation of a vaccine against parasitic infection.

Individuals with high *S. mansoni* egg counts (> 400 eggs/ml) have been found to significantly cluster ($p = .024$) in certain households (Table 2). Clustering of individuals with high *S. japonicum* egg counts was shown in one village in the Philippines ($> .025$ $p < .05$) and not in another (Tables 3 + 4). It can not be concluded that genetic factors are solely responsible for these observations but it is in these populations where genetic studies should be carried out; initially in the family clusters, then in comparative populations.

Recent studies [47] indicate that *in vitro* proliferation of T Lymphocytes by *S. japonicum* antigen may be regulated by a specific HLA haplotype. The extension of these observations into larger populations in endemic areas and investigations on the cellular mechanisms for the low responsiveness in individuals with the HLA AW24 BW52 DW 12 haplotype are currently under way.

In Chagas' disease, seropositive individuals cluster in households [40] (Table 5) as expected from our understanding of the transmission of the infection.

The genetic basis of differences in human susceptibility to *Plasmodium* infections has recently begun to be explored. The association of the presence of Duffy blood group determinants on the red cell and susceptibility to *P. vivax* [38], the higher frequency of Hb AS and the female genotype G6 PD A-/B in children without *P. falciparum* infection [6, 22] may be important factors in evaluating a vaccine against malaria.

Genetic studies to determine the association of disease patterns with specific haplotypes are relatively new but might be important in interpreting the efficacy of a vaccine which would desensitize susceptible individuals in an endemic area. In *S. mansoni* infection it has suggested that the HLA B5 locus is associated with the presence of hepatic and splenic enlargement!

TABLE 2 - *Distribution among households of persons with serological evidence of infection with* T. cruzi *in Castro Alves, Bahia, Brazil.*

No. persons infected	No. of houses observed	No. of houses expected
0	39	26.3
1	67	62.5
2	42	56.3
3	20	31.9
4	15	16.0
5	12	6.9
6	8	3.2

Significance $p = < 0.001$

Ref. « Am. Jour. Trop. Med. and Hyg. », *25*, 552-562 (1976).

TABLE 3 - *Distribution among households of persons with high intensity* (< 400 *EPG*) S. japonicum *infection in Sorsogon, Philippines.*

Village A

No. persons > 400 EPG	No. of houses observed	No. of houses expected
0	49	44.85
1	12	18.17
2	4	4.19
3	3	.78

Significance $0.25 < p < .05$

Ref. data from: « Bull. WHO », *58*, 629-638 (1980).

TABLE 4 - *Distribution among households of persons with high intensity (> 400 EPG)* S. japonicum *infection in Sorsogon, Philippines.*

Village B

No. persons > 400 EPG	No. of houses observed	No. of houses expected
0	33	33.44
1	20	19.04
2	4	4.69
3	1	.83

No significant difference p = > .50

Ref. data from: « Am. Jour. Trop. Med. and Hyg. », *25*, 285-294 (1976).

TABLE 5 - *Distribution among households of persons with high* S. Mansoni *EGG counts in Castro Alves, Bahia, Brazil.*

No. persons with > 400 EGGS/ML	No. of houses observed	No. of houses expected
0	62	52.64
1	18	25.17
2	3	8.27
3	3	2.77
4	2	0.87
5	0	
	7	3.93
6	1	
7	0	0.29
8	1	

Significance p = .024

Ref. data from: « Am. Jour. Trop. Med. and Hyg. », *25*, 285-294 (1976).

Recently the P_1 blood group has been suggested to have a positive association with susceptibility to urinary tract infections (29]. It is of interest that *Fasciola* and probably schistosomes also share this same antigen and the relationship to susceptibility to urinary tract disease due to *S. haematobium* remains to be investigated.

With all of the evidence accumulating that genetic factors influence the behaviour of parasitic infections in man, it will be important to recognize their importance during evaluation of vaccine efficacy in populations.

Cyclical characteristics of the parasite and the immune response

Within large populations, circadian or cyclical rhythms of the parasite, changing or constant transmission cycles and inherent cycles in the human immune response, all may contribute to the impact of a specific parasite vaccine.

Historically alternations in malarial parasite levels and the clinical correlates of different species have permitted the classification of the species according to the occurrence of the febrile paroxysm at the third day (*P. vivax* and *P. ovale*), fourth day (*P. malariae*), or irregularly (P. *falciparum*); all of these patterns are modified by the immune status of the infected person. Seasonal transmission of malaria has profound effects on parasitemia levels and prevalence as shown in Garki by the decreases in the dry season when transmission is lowest ([39].

Among the schistosomes, *S. japonicum* females eject the ova in bolus of 50 eggs or more while for *S. mansoni* and *S. haematobium* oviposition occurs at a constant rate. Certainly transmission is seasonal in many parts of the world. *S. japonicum* is transmitted only in the late summer in China while year around transmission occurs in the Philippines. *S. mansoni* and *S. haematobium* transmission may occur constantly or peak at the end of the rainy season in Africa. Within a 24 hour day transmission also varies according to the species and cercerial elimination from the snail.

Filaria have several different circadian rhythms in the infected host ranging from a periodic, subperiodic to highly periodic depending on the geographical location and the species [25]. In most areas transmis-

sion is relatively constant throughout the year but except in high level transmission areas the infection is acquired at night.

Parasitemia level in acute and chronic *T. cruzi* infections are so relatively low that no periodicity has been documented by current recovery methods. Transmission by the triatome vectors can occur at any time.

In none of these parasitic infections has periodicity of the immune response been established. Eosinophil counts have been observed to fluctuate in nocturnally periodic *W. bancrofti* infection and have been suggested to be related to corticosteriod levels [23].

Recently seasonal alternations in the humoral immune response have been suggested [50]. Calcium and phosphorus ions have now been shown to have a circadian 24 hrs pattern in man [37] and are involved in immune response, so perhaps this idea is not irrelevant.

In designing tactics for control of morbidity in schistosomiasis through the wide-scale use of chemotherapy, consideration has been given to these cyclical factors. Some of these factors may be relevant in the development of parasite vaccines, though I wonder if we will reach the level of sophistication as in cancer chronochemotherapy where drug administration may be synchronized with DNA repair [9].

The consistent epidemiological observations in *S. haematobium* infection fully support the view that natural immunity to this infection occurs. Research on *S. haematobium* vaccine probably offers more potential for success than with *S. mansoni.*

In conclusion evaluation of the efficacy of a parasite vaccine will require careful epidemiological evaluation of the target population, which includes consideration of the variables reviewed, prior to the administration of the vaccine as well as during the monitoring period.

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IDIOTYPE INDUCTION AS AN APPROACH TOWARD IMMUNIZATION AGAINST PARASITIC INFECTIONS

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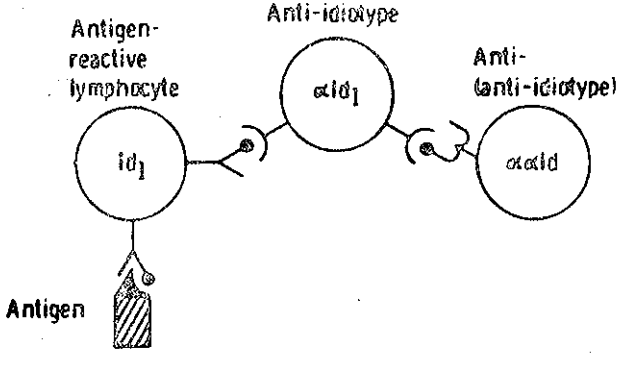
ABSTRACT

A method is described for the induction of a protective humoral immune response against parasitic infection without the use of parasite antigens. Anti-idiotype antisera are raised against monoclonal antibodies which have protective activity against the parasite. By administering the purified anti-idiotype antibodies to mice in low doses it is possible to induce a *de novo* humoral response containing the protective idiotype expressed originally on the monoclonal antibody. We have employed this approach to successfully immunize mice against infection with *Trypanosoma rhodesiense*. After injection with a mixture of mouse anti-idiotype antisera raised against three different protective monoclonals specific for a single trypanosome variant, 63% of the treated mice responded to challenge with that variant by totally or partially suppressing the parasitemia or by inducing a switch to a new variant. Serologic analysis revealed the selective expression of one of the three idiotypes in those animals displaying immunity or variant switching.

Although it is unlikely to be a feasible approach for the prophylaxis of trypanosomiasis in the field, idiotype induction may offer a strategy for immunization against some parasites where the relevant antigens are present in minute quantity or are difficult to isolate.

Idiotypes are antigenic determinants created by the unique amino acid sequences present in the hypervariable regions of individual antibody molecules. In many cases they appear to relate directly to the structural configuration of each antibody's antigen combining site. In 1973 Niels Jerne hypothesized that during the course of a conventional immune response, the increased concentration of specific antibody (i.e. idiotype) would stimulate the production of anti-idiotype antibodies which in turn would elicit the formation of anti- (anti-idiotype) antibodies (Figure 1). Such idiotype-anti-idiotype interactions would play an important role in regulating the immune response to the particular antigen initially administered [5]. An important line of evidence supporting Jerne's "network hypothesis" is the observation that the *in vivo* administration of minute quantities of anti-idiotype antibodies can modulate immune responses, that is either suppress them or actually induce them *de novo*. Indeed, using either xenogeneic, allogeneic or syngeneic anti-idiotypes it has been possible to prime mice or induce the expression of idiotype positive antibodies against a variety of antigens such as phosphorylcholine [10], streptococcal A carbohydrate, [3] NP (4-hydroxyl-3-nitrophenyl acetyl) [7], and H-2 antigens [1]. Since idiotype induction can result in the production of antibody responses in the absence of antigen, it represents an innovative and potentially powerful approach toward immunization



Elements in an idiotypic network.

FIG. 1. Jerne envisioned that the immune system consists of a « network » of idiotype-anti-idiotype interactions. In his hypothesis, the idiotypes of surface receptors on lymphocytes are recognized by a second set of anti-idiotype bearing regulatory cells. These in turn, can be regulated by a third set of anti (anti-idiotype) lymphocytes. Anti-idiotype can behave in a fashion analogous to antigen in perturbing the network.

against microbial agents and in the case of parasites could prove extremely practical in situations where the relevant target antigen(s) are difficult to isolate in suitable quantity to produce a conventional vaccine.

In this paper we describe a model system in which idiotype induction has been used successfully to immunize mice against infection with a parasite- *Trypanosoma rhodesiense*. The model will only be briefly outlined here as it is to be published in greater detail elsewhere (Sacks D., Esser K. and Sher A., manuscript in preparation).

African trypanosomiasis was deliberately chosen as the parasitic infection for these studies since in the mouse, trypanosomes are known to be sensitive to minute quantitives of humoral antibodies [9]. A clone (WRATat 1) of *T. rhodesiense* expressing one variable antigen type (VAT) was employed [2]. One of us (K. Esser) had already prepared several monoclonal antibodies specific to this variant which were shown to neutralize the parasites both *in vitro* and *in vivo* [4]. Anti-idiotypes were prepared against three of these monoclonals (7H11, 11D5, B7B1) by immunizing SJL mice with each of the purified Balb/c IgG_1 proteins. Since Balb/c and SJL mice have different immunoglobulin allotypes it was necessary to remove the anti-Balb/c allotype antibodies present in the sera by immunoabsorption with an unrelated IgG_1 myeloma protein. When analysed by competitive radioimmunoassay [8] each of the resulting absorbed anti-idiotype sera (i.e. anti-7H11, anti-11D5 and anti-B7B1) were found to be non cross reactive. Finally, following previous suggestions that the subclass of anti-idiotype may influence its regulatory effect [3, 7], IgG_1 was purified from each of the three anti idiotype sera as well as from control, normal SJL sera. The three IgG_1 anti-idiotype reagents were then administered together (i.e. in combination) into Balb/c mice in doses ranging from 250 ng-4 µg of each reagent per mouse. Twenty-four days later the animals were challenged with 100 cloned WRATat-1 parasites each and the mice bled at regular intervals in order to monitor their parasitemias and the levels of specific idiotype induced.

In the light of previous work [9] on immunity against African trypanosomes, we expected the successful induction of a specific anti-VAT response to have three alternative effects on the challenge infection: (a) complete protection of the animals (b) reduced first wave parasitemia, or (c) suppression of the original VAT in favour of a new VAT in the first wave. In fact, we have observed all three of these effects within individual experiments. The effects of reduced parasitemia and

"variant switching" are illustrated in Figure 2. In mice in which idiotype induction resulted in variant switching the peak parasitemia was delayed by 1 day (from *7* to *8* days after challenge). The presence of a new variant was indicated by the failure of the majority of the parasites in the population to react with the anti-WRATat-1 monoclonal antibodies by indirect immunofluorescence.

The results of an experiment which was analyzed in detail for all three effects are summarized in Table 1. Except for one aberrant mouse which spontaneously switched to a new variant, all of the control mice injected with normal SJL IgG gave normal infections. Thirty mice were given the mixture of anti-idiotypes in doses ranging from 250 ng to 4 μg per animal. Since no significant dose-response effects were observed over this range, the data for the different anti-idiotype treated animal groups

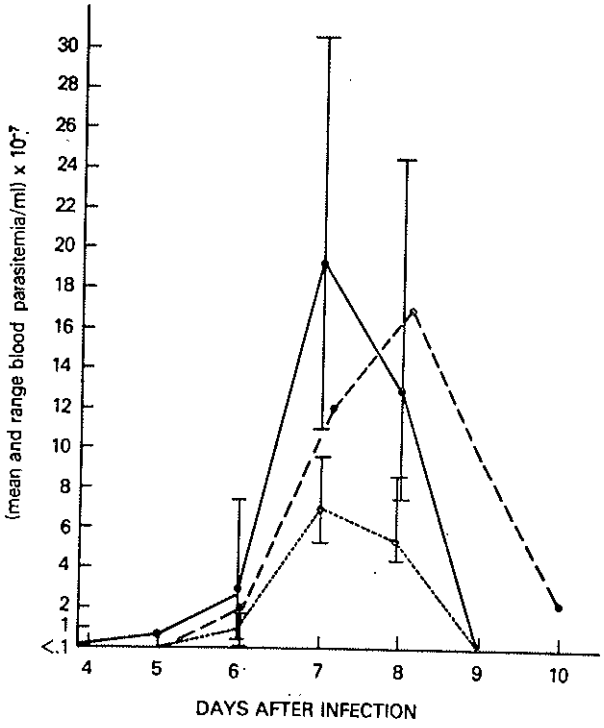


FIG. 2. Effects of anti-idiotype administration on trypanosome parasitemias in mice. The values shown are mean and range. 0——0 = control infections; 0.....0 = infections showing reduced parasitemia; 0-----0 = infections showing switching to a new variant.

were pooled (Table 1). It can be seen that 63% of these mice showed altered infections with complete protection (23%) and variant switching (27%) being the dominant effects.

In order to confirm that the immunity displayed by these animals was the result of idiotype induction, sera obtained pre-and post- challenge were analyzed for the presence of each of the three idiotypes. The results of this analysis require lengthy description. Their detailed presentation is inappropriate for the present review. However, the data can be briefly summarized as indicating a significant association of immunity with idiotype expression in the case of only one of the three idiotypes examined — the 7H11 idiotype. Thus, as can be seen in Figure 3, this idiotype was found selectively, both prior to and after challenge, in animals displaying immunity and was absent in the majority of the animals which had been treated with anti-idiotype but failed to display immunity. It can also be seen that the 7H11 idiotype is *not* induced during *normal* infection in control mice. This result is in direct contrast to the situation observed with the other two idiotypes (11D5 and B7B1), both of which were induced during normal infection (data not shown). These observations on the relationship between idiotype expression and immunity are intriguing since they suggest that the most effective anti-VAT antibody responses are the result of the expansion of minor B lymphocyte clones not induced by natural infection.

TABLE I - *Outcome of challenge infections in mice immunized with anti-idiotype antibodies.*

Animal * Group	% in each category: Normal Infection	Complete Protection	Reduced Parasitemia	Switched Variant	Altered Infection
Control (Normal SJL IgG_1)	90	0	0	10	10
Experimental (Anti-idiotype IgG_1)**	37	23	13	27	63

* The control group consisted of 10 mice, the experimental group contained 30 mice.
** Injected with 250 ng to 4 µg of each of the three anti-idiotypes/mouse.

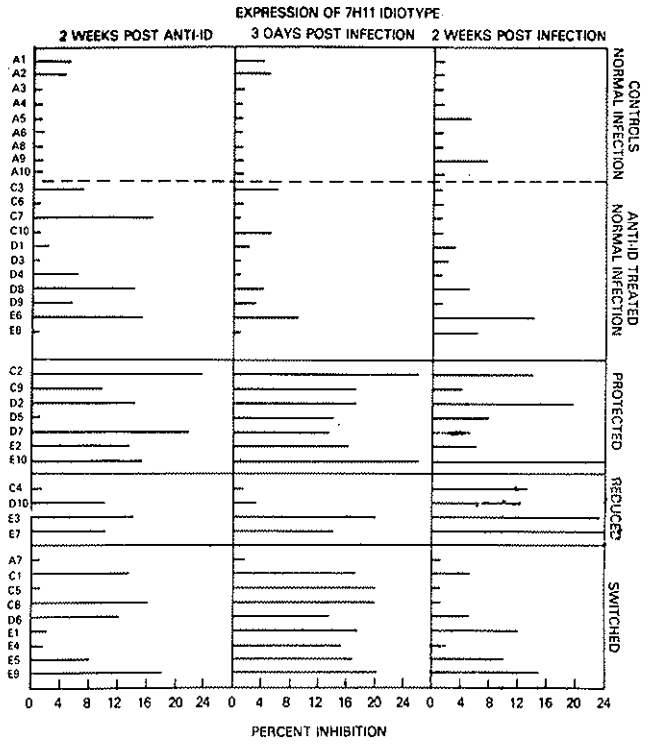


FIG. 3. Expression of 7H11 idiotype in mice grouped according to the outcome of the challenge infection.

In summary then, our results indicate that the administration of the appropriate anti-idiotypes can profoundly alter the course of *T. rhodesiense* infection in mice and that the expression of immunity in these animals correlates well with the expression of one of the three idiotypes induced. The more basic (though technically more difficult problem) of whether immunity correlates with the induction of specific anti-VAT antibody activity (as well as with specific idiotype) is currently being examined in the laboratory.

Can idiotype induction offer a general approach toward vaccination against parasites? Although the technology associated with idiotype induction is still in the early stages of development, there are indications that in the future it may be possible to use the technique to induce specific antibody in high enough levels to induce solid protection against

certain infections. For example, preliminary experiments suggest that repeated boosting with anti-idiotype or the administration of anti-idiotype coupled to carriers can greatly enhance the levels of specific idiotype produced (G. Kelsoe, personal communication). If, indeed, strong as well as long lasting antibody responses can be induced with anti-idiotypes, then the procedure could be put to use most advantageously in immunizing against parasites whose target antigens are difficult to isolate in sufficient quantity to permit conventional vaccine development. Immunization against malaria sporozoites [11] (a life-cycle form which can be obtained only in minute amounts) is an example of an important problem which might benefit from this approach. The recent development of techniques for generating human monoclonal antibodies [6] suggests that in the future monoclonal protective antibodies as well as anti-idiotype antibodies could be generated from human lymphocytes thereby allowing the use of isologous immunoglobulins in the induction of idiotypes in man.

Finally, in addition to its modulatory effect in inducing immune responses it should be remembered that the *in vivo* administration of anti-idiotype antibodies under certain conditions can also suppress the expression of idiotypes [3]. In this regard, anti-idiotypes could potentially be put to use in the suppression or prevention of parasite induced immunopathology. In summary, because of their possible relevance to both immunization and immunologic intervention, the idiotypes induced by parasite infections are clearly worthy of extensive investigation. The study of their expression and regulation is likely to be a major emphasis in immunoparasitologic research in coming years.

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VACCINATION AGAINST MALARIA: USE OF MONOCLONAL ANTIBODIES FOR THE CHARACTERIZATION OF THE PROTECTIVE ANTIGENS

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Herein we summarize the recent progress in our understanding of the immune response to malaria parasites, which has been greatly facilitated by the development of monoclonal antibodies against these parasites. The emphasis of the research in this area has been on the identification and characterization of the antigens which induce a protective response in the immunized host; i.e., those antigens which in the future might be used for a malaria vaccine. This represents a clear departure from earlier approaches to malaria immunization, which were based primarily on the use of intact, attenuated parasites as immunogen.

Earlier investigation had shown that immunization with the "free" extracellular stages of Plasmodia, namely, sporozoites, merozoites, and gametes, induces the most effective immune responses (Carter, 1980; Cochrane, 1980; Siddiqui, 1980). Monoclonal antibodies against these various developmental stages were therefore developed in several laboratories, for the purpose of characterizing "protective antigens".

Identification and characterization of merozoite antigens

With regard to asexual blood stages, particularly merozoites, three different malaria species, *P. yoelii*, *P. knowlesi* and *P. falciparum*, have been used to obtain monoclonal antibodies.

A series of hybridomas were obtained by Freeman (1980), upon immunization of mice with blood stages of *P. yoelii*, a rodent malaria parasite.

Their reaction with infected red blood cells resulted in several distinct patterns of immunofluorescence, suggesting that they recognize different antigens. These hyhridomas produced a variety of different subclasses of IgG. Two of these antibodies displayed biological activity *in vivo*, since their passive transfer to *P. yoelii*-infected mice altered the course of infection, resulting in a lower, more transient level of parasitemia, and survival of the animals. This effect, however, was much less marked than that produced by passive transfer of hyperimmune serum.

In a recent communication, Freeman (1981), reported that immunization of mice with the corresponding high molecular weight antigen (240,000 daltons), resulted in partial protection of mice against *P. yoelii* challenge. This antigen was purified by affinity chromatography using the protective monoclonal antibody.

Epstein (1981) recently reported the production of several monoclonal antibodies which react with the surface of merozoites of *P. knowlesi*, a parasite of monkeys. These antibodies differ in their capacity to induce merozoite agglutination. In an *in vitro* assay it was found that only two of these hybridomas, those which induce strong merozoite agglutination, significantly decreased merozoite invasion of red blood cells. A rather high concentration of monoclonal was required to produce decreased merozoite invasiveness. The hyperimmune serum must also be present in high concentration in order for complete inhibition of merozoite invasion to occur.

A series of monoclonal antibodies reacting with merozoites and schizonts of *P. falciparum*, a human malaria parasite, have been obtained by Perrin (1981). These hybridomas immunoprecipitate polypeptide chains of different molecular weights. Some of these monoclonals were capable of inhibiting parasite reinvasion and intra-erythrocytic development in an *in vitro* culture system. One of these inhibitory monoclonal antibodies was raised by immunization of mice with blood stages of *P. berghei*, a rodent malaria parasite.

Identification of gamete antigens

This research has been pursued in an avian malaria system, using monoclonal antibodies produced by immunization of mice with *P. gallinaceum* gametocytes. Male and female gametes were shown to share certain surface antigens, since a single monoclonal antibody was observed to produce fluorescence with both sexual stages.

Two of these monoclonal antibodies induce a moderate degree of microgamete agglutination. This agglutination was strongly enhanced by combining the two monoclonal antibodies of different specificity. Combining these two monoclonals also produced a clear potentiation of their biological activity. It resulted in microgamete immobilization, a significant decrease in gamete fertilization, and as a result, blocking of malaria transmission (Rener, 1980).

Transmission-blocking immunity, through antibody-mediated microgamete immobilization, has also been shown to occur in rodent and simian malaria, by immunizing mice with *P. yoelii* blood stages (Mendis, 1979), and rhesus monkeys with gametocytes of *P. knowlesi* (Gwadz, 1978).

Identification and characterization of sporozoite surface antigens

Monoclonal antibodies, and identification of tbe respective antigens, have been obtained for sporozoites of different species of rodent malaria (Yoshida, 1980; Yoshida, 1982); two simian malarias, *P. knowlesi* (Cochrane, 1981) and *P. cynomolgi* (Hii, unpublished results); and the sporozoites of human malaria, *P. falciparum* and *P. vivax* (Nardin, unpublished results).

A monoclonal antibody, produced against *P. berghei* sporozoites, has been found to be directed against a surface protein of molecular weight 44,000, designated Pb44, which is evenly distributed over the entire surface of mature salivary gland sporozoites (Yoshida, 1980). This polypeptide is considered to be a differentiation antigen, since it is either absent, or present only in small amounts on earlier developmental stages, namely, oocyst sporozoites. Pb44 persists and can be detected on early, but not on late, liver developmental stages, and is absent from blood forms of *P. berghei* (Aikawa, 1981). This antigen has been postulated to have an important role in sporozoite infectivity and development, particularly in its interaction with, and subsequent internalization by host cells. The results of recent *in vitro* experiments tend to support this view (Hollingdale, 1981).

Incubation of sporozoites of *P. berghei* with either intact monoclonal antibody (3D11), an IgG1, or its F(ab) fragments, abolishes sporozoite infectivity; i.e., induces sporozoite neutralization *in vivo* (Potocnjak, 1980) and *in vitro* (Hollingdale, 1981). Furthermore, the passive transfer of as little as 10 μg of this purified antibody to mice renders them completely resistant to sporozoite challenge.

Metabolic labeling of *P. berghei* sporozoites with ^{35}S-methionine, followed by immunoprecipitation of the solubilized parasite extract, reveals the presence of two additional, internal polypeptide chains, of molecular weights 54,000 and 52,000 (Pb54 and Pb52), which share an antigenic determinant with the surface antigen Pb44. These three polypeptides (Pb54, Pb52 and Pb44) constitute a major component of *P. berghei* sporozoite extracts. In fact, the incubation of a methionine-labeled sporozoite extract with the monoclonal antibody 3D11 results in immunoprecipitation of 10 to 15% of the metabolically-labeled protein (Yoshida, 1981).

A series of monoclonals has been obtained which react with a surface antigen of *P. knowlesi*, a primate malaria parasite. Of nine monoclonals, seven immunoprecipitated the same proteins from extracts of sporozoites labeled with ^{35}S-methionine. At least four of these purified monoclonals (all IgG1), as well as their F(ab) fragments, abolish sporozoite infectivity (Cochrane, 1981).

Five of these anti-*P. knowlesi* monoclonals cross-react with a surface antigen of sporozoites of *P. cynomolgi*, another simian malaria parasite, and one also reacts with a surface antigen of *P. falciparum* sporozoites. In each of these instances, the shared determinant on the cross-reacting sporozoites is located on a polypeptide chain of molecular weight different from that of *P. knowlesi*.

An anti-*P. falciparum* hybridoma was produced by immunization of mice with sporozoites of West African (Gambian) origin. It was found to react intensively with a surface antigen (molecular weight approx. 67,000) of sporozoites of *P. falciparum* obtained in Thailand. The same band was immunoprecipitated by this monoclonal antibody, by the immune serum of a human volunteer immunized and protected against *P. falciparum* sporozoite challenge, and by the sera of some individuals living in a *P. falciparum*-endemic area, as well as by the cross-reacting anti-*P. knowlesi* hybridoma (Nardin, unpublished results).

Incubation, with this monoclonal antibody, of *P. falciparum* sporozoites of Thai origin, induced a considerable but not complete loss of sporozoite infectivity, as detected by inoculation into splenectomized chimpanzees.

The findings with the anti-*P. vivax* monoclonal antibodies are fundamentally analogous. The molecular weight of the surface antigen detected on these sporozoites is distinct, but the precipitation pattern in SDS gel of the ^{35}S-methionine-labeled *P. vivax* sporozoite(s) (3 polypeptide chains), is

similar to that of the other sporozoite species. Furthermore, all these sporozoite surface antigens have low but distinct isoelectric points (range between 4.0-5.0). One of the recently obtained anti-*P-cynomolgi* monoclonal antibodies also reacts with a *P. vivax* surface antigen (Hii, unpublished observations).

Preliminary data on the peptide maps of several of these sporozoite antigens indicate that there are considerable homologies, which are greatest between the simian malarias (Santoro, unpublished results).

These structural and immunological findings suggest that these polypeptides, their precursors and the cross-reacting molecules, constitute a family of circumsporozoite proteins with shared structure and function. This, as well as the fact that these polypeptides constitute a major sporozoite component, may render easier the task of cloning the corresponding sporozoite gene(s), aiming at the production of protective antigens by recombinant DNA technology.

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CELLULAR IMMUNE RESPONSES AND IMMUNOREGULATION DURING HUMAN SCHISTOSOMIASIS

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ABSTRACT

Cell-mediated immune responsiveness during human schistosomiasis was studied using peripheral blood mononuclear cells (PBMN) obtained from patients and *in vitro* culture techniques. Responsiveness was assayed in regard to schistosomal-derived antigenic preparations from eggs (SEA), adult worms (SWAP), and cercariae (CAP), as well as phytohemagglutinin-P and a *Candida albicans* extract. The PBMN commonly observed with chronically infected ambulatory patients are good to PHA, poor to SEA, strong to SWAP, good to CAP and good to *C. albicans* extract. The SEA-, SWAP-, and CAP-induced responses are usually suppressed in the presence of sera from patients with schistosomiasis. This schistosome-specific sero-suppression is often observed in conjunction with another form of schistosome-specific suppression mediated by an adherent/phagocytic cell. Lymphocyte blastogenesis studies conducted longitudinally pre- and post-chemotherapy with praziquantel demonstrate that anti-schistosomal immune responses are almost always augmented by successful chemotherapeutic treatment. Thus far such increased spe-

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cific responsiveness has been observed at 1 and 3 months following treatment. The majority of chronically infected patients do not produce the lymphokine activity termed Mitogenic Factor upon *in vitro* exposure to SEA, SWAP or CAP. However, PBMN from several subjects, who approximately 20 years previously had been successfully treated for their schistosomiasis, can be stimulated by these preparations to produce Mitogenic Factor activity. It appears that during active chronic infection effector functions such as antigen-induced lymphokine production may be even more heavily regulated than the antigen recognition phenomenon represented by lymphocyte blastogenesis.

Introduction

Chronic human schistosomiasis is usually characterized by a relatively stable host-parasite relationship (Warren, 1973). The establishment and maintenance of this situation has been seen to coincide with the expression of both strong anti-schistosomal immune responses and specific and non-specific immunoregulatory systems (Phillips and Colley, 1978; Colley, 1981). The majority of studies dealing with such cellular reactions during clinical schistosomiasis have utilized *in vitro* peripheral blood mononuclear cell (PBMN) or whole blood blastogenesis assays against various heterogeneous schistosomal antigenic preparations obtained from eggs (SEA), adult worms (SWAP) and cercariae (CAP) (Colley *et al.*, 1977a; Ottesen *et al.*, 1978; Abdel-Salam *et al.*, 1981). Although these crude antigenic mixtures undoubtedly contain some shared and cross-reacting moieties it is remarkable that they commonly each induce characteristic degrees of responsiveness in PBMN cultures. Thus in schistosomiasis mansoni SEA-induced blastogenesis is usually strongest early on in infections and decreases dramatically with the duration of infections (Ottesen *et al.*, 1978; Colley *et al.*, 1977a). Parallel studies of SWAP and CAP responsiveness demonstrate the development of regulatory mechanisms which govern them, but in general a greater degree of PBMN reactivity to these materials is commonly observed as infections became established (Colley *et al.*, 1977a; Colley *et al.*, 1977b; Ellner *et al.*, 1980; Ottesen, 1979; Ottesen and Poindexter, 1980; Reiner *et al.*, 1979; Todd *et al.*, 1979). The present investigations report similar findings in ambulatory Egyptians with schistosomiasis mansoni and hematobia. Furthermore, increased PBMN responsiveness to all 3 antigenic prepa-

rations are demonstrated in this population following successful chemotherapeutic treatment with praziquantel. From another group of studies, on Brazilian patients with schistosomiasis mansoni, we now report on PBMN schistosomal antigen-induced production of the lymphokine (LK) Mitogenic Factor. Mitogenic Factor (MF), as assayed by its ability to stimulate blastogenesis in PBMN cultures (Rocklin *et al.*, 1974), is often not produced by SEA, SWAP, or CAP exposed PBMN from chronic schistosoma patients. However, it appears that former patients who were successfully treated years previously for their schistosomiasis actively express this cell-mediated function.

Materials and methods

Patients. Patients were selected on the basis of clinical and parasitological data obtained by the Bilharziasis Research Project and the Centro de Pesquisas "René Rachou". Informed consent was obtained, either in English, Arabic, or Portuguese, before the inclusion of each subject in the study.

Antigenic preparations and mitogens. Phytohamagglutinin P (PHA) (Difco Laboratories, Detroit, MI) was used at 2.5 μl/ml of culture fluid. The soluble schistosomal antigenic preparations were derived from either *Schistosoma mansoni* eggs (SEA), adult worms (SWAP), or cercariae (CAP), as previously described (Colley *et al.*, 1977a). Their protein content was determined by the method of Lowry (1951) and they were used at a final concentration of 10-20 μg total protein/ml culture fluid. *Candida albicans* extract (Hollister-Stier Laboratories, Atlanta, GA) was dialyzed against saline and RPMI 1640 (GIBCO, Grand Island, NY) and used at 40 μl/ml of culture fluid as an unrelated, non-schistosomal antigenic preparation (Colley *et al.*, 1977b; Todd *et al.*, 1979).

Cell preparation and culture conditions. Peripheral blood mononuclear cell populations (PBMN) were obtained by a 400 × g centrifugation at 18°-20°C for 40 min of whole, heparinized blood over a Ficoll-diatrizoate mixture (LSM, Litton Bionetics, Inc., Kensington, MD) and were subsequently washed 3 times in MEM-S (GIBCO) and once in RPMI 1640 (Colley *et al.*, 1977a; Colley *et al.*, 1978).

Non-adherent/Non-phagocytic (NA/NP) cell populations were prepared from PBMN following 1 hr incubation of 25×10^6 PBMN in

6.6 ml of RPMI 1640 —10% NHS plus 10 mg of carbonyl-iron, in 60 × 15 mm, Type 3030 plastic tissue culture dishes (Falcon, Oxnard, CA) at 37°C. NA/NP cells were resuspended over a magnet, collected, washed twice and cultured in parallel with unseparated PBMN (Todd *et al.*, 1979).

For PBMN blastogenesis assays cells were cultured in triplicate cultures in a medium consisting of 91% RPMI 1640, 1% L-glutamine (stock of 200 mM), 3% antibiotic-antimycotic (stock of 100 units of penicillin, 100 mM streptomycin and 25 μg Fungizone per ml) (GIBCO), and 5% fresh frozen, heat-inactivated (56°C/30 min) human, AB^+ serum (NHS). In some experiments the effect of using the patient's autologous serum as the medium supplement was contrasted with the use of NHS. Antigen or mitogen was added in an aliquot of RPMI 1640 at concentrations determined to be generally optimal in this system (see above). Cultures were in 0.2 ml volumes, in flat-bottom microtiter tissue culture plates (Linbro, McLean VA) and were maintained at 37°C in a humid 5% CO_2, 95% air environment. The number of PBMN per culture was 250,000 and they were tested for tritiated thymidine (^{3}H-TdR) incorporation by the addition of 0.5 μCi/well during the last 6 hr of a 6 day culture period and scintillation counting. Thymidine (Sp. Act. 2.0 Ci/mM) was purchased from New England Nuclear (Boston, MA).

For Mitogenic Factor (MF) production 2 ml cultures of 6×10^6 PBMN were incubated in 16 × 125 mm glass tubes (Brockway, American Scientific, Stone Mountain, GA). The final medium was the same as for the blastogenesis assay given above and the antigen concentrations were 15 μg, 15 μg and 20 mg per ml for SEA, SWAP, and CAP, respectively. After an 18-20 hr incubation, antigen-exposed and control (unexposed) cultures were centrifuged at 200 × g/10 min/6°C, their culture fluids removed and discarded, and resuspended in freshly prepared complete medium. Antigens were not added, but the cultures were reincubated. After an additional 48 hr the culture supernatant fluids were collected (300 × g/10 min/6°C) and stored in polypropylene tubes (N° 2063, Falcon) at -20°C.

Culture supernatant fluids were assayed for MF activity using a modification of the above PBMN blastogenesis assay. PBMN cells (200,000) were cultured in 150 μl of culture medium (89.5% RPMI 1640, 1.75% L-glutamine, 3.25% antibiotic-antimycotic, 5.05% NHS) to which was added 50 μl of the culture supernatant fluid to be tested for MF activity. Each experimental fluid (antigen-stimulated) was tested

against its own control fluid (supernatant fluid from the same cells cultured in parallel but without antigenic exposure). MF assay cultures were exposed to 1.0 μC/ml ^{3}H-TdR for the last 18 hr of a 6 day culture period.

Means of the triplicate counts per minutes (CPM) were determined and differences between paired or grouped means were analyzed for significance by tbe appropriate Student t-test; significance = $p < 0.05$. Data are presented as CPM, Experimental (stimulated) CPM-Control (unstimulated) CPM (E-C), or Experimental CPM/Control CPM (E/C).

Chemotherapy. Praziquantel was administered orally using a single dose of 40 mg/kg body weight. Patients were selected, treated and followed by the clinical staff of the Bilharziasis Research Project. Pre- and post-treatment parasitological examinations were done on all patients.

Results

PBMN blastogenesis assay. We have previously demonstrated, studying St. Lucian patients with schistosomiasis mansoni (Colley *et al.*, 1977a; Todd *et al.*, 1979) that most ambulatory, chronically infected patients respond very well to SWAP, and moderately well to CAP, PHA and Candida extract, but rather poorly to SEA. These general observations were reaffirmed with Egyptian patients who were doubly infected with *S. mansoni* and *S. haematobium* when tested against *S. mansoni*-derived antigens (Figure 1). The 10 patients whose PBMN responses are summarized in Figure 1 had a mean age of 29 years (range 15-55), and arithmetic mean egg counts in stool and urine of 71 (range 3-186) and 53 (range 2-197), respectively. None were hepatosplenic.

Serosuppression. Comparisons of Egyptian PBMN responses in media supplemented with 5% of either NHS or autologous serum revealed the schistosomal antigen-specific serum-mediated regulatory condition we have referred to as serosuppression (Colley *et al.*, 1977b; Todd *et al.*, 1980). Figure 2 illustrates the serosuppressive effects of autologous serum in PBMN cultures from two of our Egyptian patients. Consistent changes are not effected by autologous serum on the control, PHA, or Candida extract values. However, the almost non-existent SEA response, as well as those strong responses to SWAP and CAP were uniformly suppressed by patients' sera.

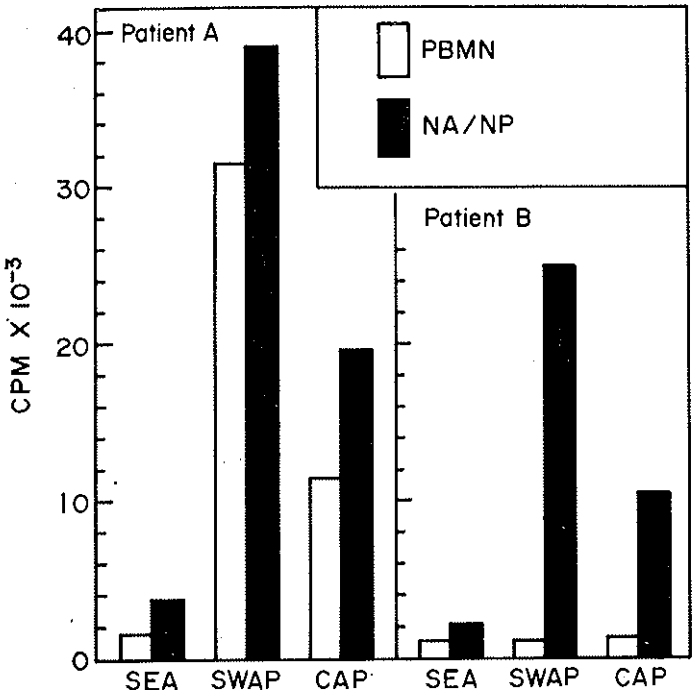

FIG. 1 — Tritiated thymidine incorporation by PBMN from 10 patients infected with *S. mansoni* and *S. haematobium*. Cultures were exposed to medium alone (control), the mitogen phytohemagglutinin (PHA), extracts from *S. mansoni* eggs (SEA), adult worms (SWAP), or cercariae (CAP), or an extract of *Candida albicans* (*C. albicans*) for 6 days.

NA/NP responsiveness. As we reported when studying St. Lucian schistosomiasis (Todd *et al.*, 1979) after removal of the adherent/phagocytic cells from PBMN suspensions the resultant NA/NP cell population usually responds more strongly to the schistosomal preparations. This is again demonstrated with two Egyptian cases in Figure 3. As with serosuppression the Control, PHA and Candida extract responses were only inconsistently altered to a minor degree by adherent/phagocytic cell removal.

Pre-and post-treatment responsiveness. Individual patients were longitudinally studied for their PBMN responsiveness prior to and following successful chemotherapeutic treatment with praziquantel. The success of treatment was ascertained by parasitological examination of multiple stool and urine specimens. PBMN cultures were done immediately prior to

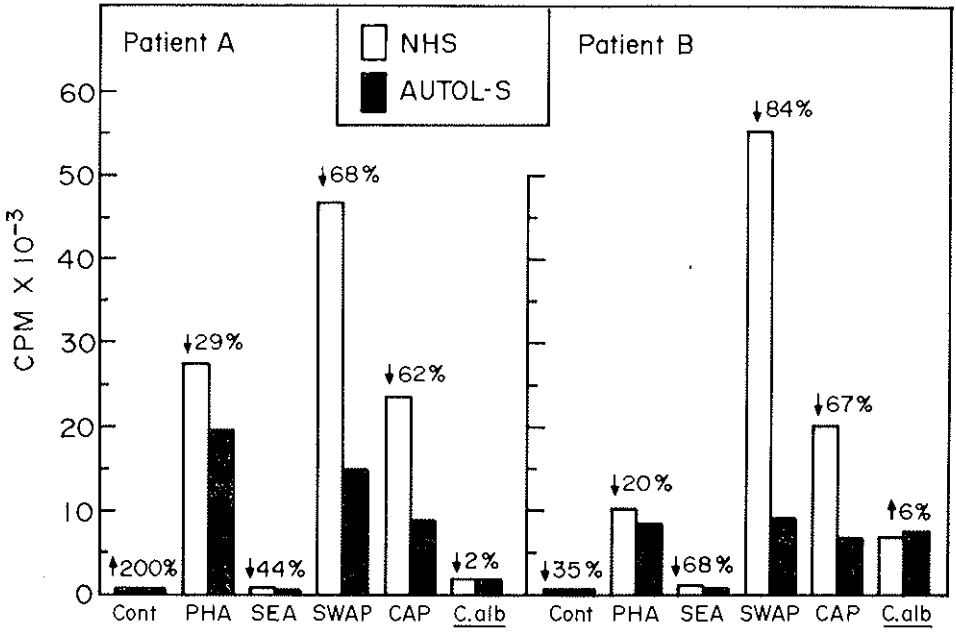

FIG. 2 — Tritiated thymidine incorporation by PBMN from 2 individual patients. Patient A (55 years of age) had *S. mansoni* (19 eggs per gram of stool) and *S. haematobium* (16 eggs per 10 ml urine). Patient B (21 years of age) currently had only *S. mansoni* infection (14 eggs per gram of stool). Culture medium was either supplemented with 5% normal human serum (NHS) or 5% of the patient's own autologous serum (AUTOL-S). The percent change CPM found in AUTOL-S supplemented cultures relative to NHS supplemented culture is indicated above each comparison. Abbreviations as in Figure 1.

treatment and either 1 month or 3 months following treatment. Table I shows that a group of 11 patients studied at 1 month following therapy had significant increases in their SWAP- and CAP-induced reactivities but their control CPM and responses to PHA, Candida extract and SEA were unaltered by cure of their schistosomiasis. Table II presents similar data from a group of 23 patients prior to and at 3 months after chemotherapy. The data for control CPM, PHA and Candida extract reactivities remain unchanged as was seen at the 1 month time point (Table I). However, at the 3 month period this group shows that significant increases were observed in the responses to each of the three schistosomal preparations, now including SEA. Although the SEA responsiveness was crearly augmented by therapy it still remained considerably less than that stimulated by either SWAP or CAP.

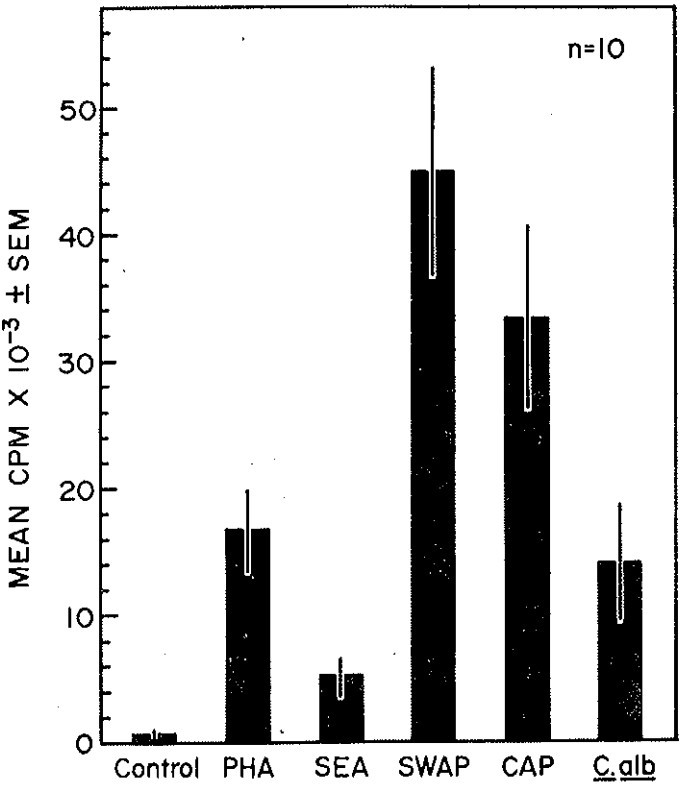

FIG. 3 — Tritiated thymidine incorporation by PBMN and the non-adherent/non-phagocytic (NA/NP) population from PBMN of 2 individual patients. Patient A (hepatosplenic) had 59 eggs per gram of stool and Patient B (19 years of age and asymptomatic) had 213 eggs per gram of stool. Neither were harboring *S. haematobium* at the time of examination.

Production and assay of Mitogenic Factor. The lymphokine (LK) termed Mitogenic Factor (MF) is produced by T lymphocytes, and is capable of stimulating, by itself, some degree of blastogenesis and ^{3}H-TdR incorporation in fresh cultures of unseparated PBMN (Rocklin *et al.*, 1974). This type of LK activity was not produced when PBMN from Control subjects (never exposed to, or infected with, schistosomes) were exposed to SEA, SWAP, or CAP (Table III). However, SEA, SWAP and CAP exposure of PBMN from two subjects who had previously had *S. mansoni* infections but had been successfully treated between 17 and 25 years previously did result in the production of MF activity (Table III). This activity was successfully assayed on normal, unrelated PBMN (Table III)

TABLE I — *Mean Responsiveness of 11 Patients Before and 1 Month After Praziquantel Treatment.*

	Pre-Treatment	*1 Month Post-Treatment*
Control (cpm ± SEM)	775 ± 131	1151 ± 481
Exogenous Stimulation (E/C ± SEM)		
PHA-P	21.7 ± 5.4	21.6 ± 5.2
C. albicans Ext	4.7 ± 1.0	3.7 ± 1.0
SEA	2.0 ± 0.4	3.0 ± 0.7
SWAP	7.3 ± 2.5	17.7 ± 4.2*
CAP	3.4 ± 0.8	9.4 ± 2.4*

* Group data were analyzed by individual paired Student's t-tests. Means noted by asterisks indicate significant differences ($p < 0.05$) between pre-treatment and 1 month post-treatment responses.

TABLE II — *Mean Responsiveness of 23 Patients Before and 3 Months After Praziquantel Treatment.*

	Pre-Treatment	*3 Months Post-Treatment*
Control (cpm ± SEM)	665 ± 108	667 ± 117
Exogenous Stimulation (E/C ± SEM)		
PHA-P	43.6 ± 13.5	57.9 ± 13.3
C. albicans Ext	4.3 ± 0.6	4.4 ± 0.9
SEA	2.1 ± 0.4	5.1 ± 1.2*
SWAP	6.3 ± 1.3	24.9 ± 5.2*
CAP	4.5 ± 0.8	10.7 ± 2.2*

* Group data were analyzed by individual paired Student's t-tests. Means noted by asterisks indicate significant differences ($p < 0.05$) between pre-treatment and 3 month post-treatment responses.

TABLE III — *Mitogenic Factor Assay of Control and Schistosomal Antigen-Stimulated PBMN Culture Supernatant Fluids (Lymphokines, LK).*

Source of PBMN	*Counts Per Minute and (E/C) Values*			
Control Subjects	*Control-LK*	*SEA-LK*	*SWAP-LK*	*CAP-LK*
Nº 1	80	86 (1.1)	84 (1.1)	77 (1.0)
Nº 2	311	——	191 (0.6)	——
Treated Subjects				
Nº 1 a*	1615	10999 (6.8)	14264 (8.8)	4199 (2.6)
b	414	1844 (4.5)	2894 (7.0)	2363 (5.7)
c	433	——	1640 (3.8)	——
Nº 2	162	384 (2.4)	628 (3.9)	258 (1.6)
S. mansoni Patients				
Nº 1	192	150 (0.8)	160 (0.8)	——
Nº 2	151	163 (1.1)	275 (1.8)	——
Nº 3	1320	848 (0.7)	893 (0.6)	——
Nº 4	683	1784 (2.6)	2340 (3.4)	——

* a, b, and c indicate separate LK preparations from Treated Subject Nº 1 on 3 different days, and assayed separately.

and even on PBMN from some schistosomiasis patients (preliminary data, not presented). PBMN from most ambulatory Brazilian patients with active *S. mansoni* infection (approximately 70%) did *not* respond to SEA, SWAP, or CAP by the production of MF activity (Table III). Of those four patients presented in Table III three did not respond while one did. This small group is representative of more than 40 patients so studied thus far. Table IV presents evidence from four other patients that this unresponsiveness in regard to antigen-induced MF production often occurred with PBMN from patients whose same PBMN responded very will to the same antigen in regard to ^{3}H-TdR incorporation. To date we have not noted any clinical or parasitological correlations to those approximately 25% of patients who do produce MF activity, nor do we see relationships as to why some patients have positive blastogenesis and negative MF

TABLE IV — *Lack of Correlation Between Positive PBMN Blastogenesis to SWAP and SWAP-Induced Mitogenic Factor (MF) Production.*

S. mansoni Patient	Blastogenesis Response (E-C; CPM)	MF Production (E/C)
Nº 1	22692	1.3
Nº 2	37736	1.8
Nº 3	13059	0.8
Nº 4	21353	0.6

production while others are positive in both aspects. Thus far examples of positivity for MF production in the absence of blastogenesis have been very few and must still be considered to be questionable.

DISCUSSION

In the last 5 years it has become apparent that strong schistosomal antigen-specific cell-mediated immune responses are generated during the course of human schistosomiasis. It has, at the same time become clear that equally effective specific and non-specific immunoregulatory events are manifested (Colley, 1981). It seems obvious that the balance of these systems, coupled with other homeostatic controls, aids in the establishment and maintenance of the long term, stable relationship which characterizes most human-schistosome interactions.

If that is true, then possibly shifts or imbalances in the reported systems may account for the development of the severe clinical end of the spectrum, hepatosplenism, urological symptomatology, and in some settings polyposis. Thus far we have been unsuccessful in correlating the various immunoregulatory conditions described with any clinical or parasitological differences in patients (Colley *et al.*, 1977a; Colley *et al.*, 1977b; Colley *et al.*, 1978; Colley *et al.*, 1979; Todd *et al.*, 1979; Todd *et al.*, 1980; Todd *et al.*, 1981). This has been similar to the results of Ottesen *et al.* who have also primarily studied ambulatory schistosomal patients rather than hospital-based patient populations with severe forms of the disease

(Ottesen *et al.*, 1978; Ottesen, 1979; Ottesen and Poindexter, 1980). Splenic suppressor (Ellner *et al.*, 1980) and splenic adherent helper cells (Reiner *et al.*, 1979) have been noted in severe cases. A relationship has been reported between low blastogenesis responsiveness to SWAP and high (> 600 eggs/gram) intensity of infection (Ellner *et al.*, 1981), and a significant association between the development of hepatosplenomegaly and the inheritance of the histocompatibility antigens HLA-A1 and HLA-B5 has been noted (Abdel-Salam *et al.*, 1979).

Several groups have observed the type of augmentation of anti-schistosomal cell responses presented herein following chemotherapy. From two weeks to several months after effective therapy (with Hycanthone, Niridazole or Praziquantel) these specific responses are markedly increased (Ottesen *et al.*, 1978; Ottesen and Poindexter, 1980; Abdel-Salam *et al.*, 1981). The reason for this augmentation has yet to be determined, but it could well be either due to a boosting of the patient's immune system following antigenic release due to portal intravascular worm death or (more appealing immunologically) due to alterations in immunoregulatory events. It would seem that continued study of this point, perhaps in relationship to chemotherapy and subsequent rates of reinfection, would be desirable.

Lymphokine studies have been few in human schistosomiasis. PBMN from patients with either *S. mansoni* (Kazura *et al.*, 1975) or *S. haematobium* (Wadee and Sher, 1980) infections can be seen to respond to their homologous egg antigens or intact eggs, respectively, by the production of the lymphokine called eosinophil stimulation promoter (ESP). In the current study it was seen that the majority of active schistosomiasis patients did not respond to SEA, SWAP or CAP by the production of MF activity. This was true even if they did respond with positive lymphocyte blastogenesis. Interestingly, former patients (subjects who had had schistosomiasis years previously and had undergone successful chemotherapy) did respond by producing MF activity. Such subjects can almost always be counted on to respond most vigorously to SEA, SWAP and CAP in the blastogenesis assay (Colley, unpublished observations). This is even true if their therapy was as much as 30 years previously. It would seem that following successful chemotherapy there is an eventual loss of most regulatory systems while the positive memory arm of the immune system remains intact. It has yet to be seen if this responsiveness which is observed late after treatment is related to the initial augmentation of specific responses seen as early as 2 weeks after chemotherapy.

ACKNOWLEDGEMENTS

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DEVELOPMENT OF RESISTANCE BY *SCHISTOSOMA MANSONI* SCHISTOSOMULA AGAINST ANTIBODY MEDIATED KILLING INDUCED BY SERUM FACTORS AND CONCANAVALIN A

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ABSTRACT

Fresh schistosomula can be killed *in vitro* by an antischistosome antibody (lethal antibody) in tbe presence of complement. When cultured in a chemically defined medium (NCTC-135) they become gradually insensitive to the killing effect of the lethal antibody. Addition of 200 μg of partially purified serum factor (PPSF) from rabbit or 5 μg of Concanavalin A (Con A) to the culture medium resulted in a further increase in the number of parasites insensitive to the lethal antibody over a shorter period of incubation. This effect was not due to an anticomplementary effect of the PPSF or Con A since guinea-pig serum (source of complement) pre-incubated with either of these substances for lh at room temperature, did not lose its cytotoxic activity. Binding of anti-parasite antibody to scbistosomula surface as measured by indirect fluorescence and ^{125}I labelled IgG did not show significant change after pre-incubation with Con A or PPSF. The protection induced by Con A schistosomula is partially prevented by 1.3×10^{-4} mmoles of α-methyl-mannoside added to the culture medium together with 5 μg of Con A. A progressive reduction of the protection was also observed as the time of culture preceding Con A addition increased.

When 10,000 schistosomula were treated with 50-500 μg of trypsin for 30 min, high molecular weight components of the parasite tegument

were hydrolyzed as shown by gel electrophoresis of iodine labelled tegument. Following trypsin treatment the schistosomula become more sensitive to the lethal antibody and the parasite loses its capacity to develop resistance to andibody-mediated killing in presence of Con A. Incorporation of labelled amino-acid into the tegumental proteins was stimulated by PPSF but not affected by Con A. Gel electrophoretic patterns of labelled tegument from fresh and resistant schistosomula are very similar.

Introduction

Following transformation from cercariae, *Schistosoma mansoni* schistosomula become gradually insensitive to the lethal effect of immune sera and complement *in vitro* (Clegg & Smithers, 1972). It is believed that the acquisition of host molecules onto the surface of the parasite may prevent the schistosomula from being recognized as a foreign (Clegg & Smithers, 1972; McLaren *et al.*, 1975). In fact several different types of host molecules have been shown to be acquired by the schistosomula, including glycolipids which demonstrate blood group specificity (Clegg *et al.*, 1971; Goldring *et al.*, 1976), Forssman-like antigens (Dean & Sell, 1972) and gene products of mouse major histocompatibility complex (Sher *et al.*, 1978). On the other hand, it has been shown that fresh schistosomula can develop resistance to lethal antibody when cultured in a chemically defined media (Dean, 1977; Novato Silva *et al.*, 1980). We have confirmed this observation and in further studies we have demonstrated that the rate of acquisition of protection by schistosomula against antibody and complement may be stimulated by serum factors or Concanavalin A. Furthermore, efforts have been made to detect protein or glycoprotein alterations in the tegument of the parasite during the period of time in which it becomes insensitive to the effect of lethal antibody.

Materials and methods

Schistosomula preparation

Cercariae of *S. mansoni* (L. E. Strain, Belo Horizonte, Brazil) were obtained from laboratory-reared *Biomphalaria glabrata* and concentrated

as described by Gazzinelli *et al.* (1973). Packed cercariae were resuspended in 15 ml glass conical tubes, each containing 2.0 ml ice-cold Elac (Earle's saline with 0.5% lactalbumin hydrolysate and 0.1% glucose, pH 7.4) with 200 units/ml of penicillin and 200 µg/ml streptomycin. Each tube was agitated for 45 sec. on a Vortex Genie rotor (Bender & Hobein AG, Zürich, Switzerland) and the suspension transferred to 2 tubes, each containing 5.0 ml Elac (final volume 6 ml/tube). After 6-7 min the tail-rich supernatant was decanted and the bodies were washed twice in 6.0 ml Elac and collected in a vial. The cercarial bodies (cerca 50,000/vial) were incubated at 37°C in Elac + 20 mM Hepes for 90 min under gentle agitation. During this period the cercarial bodies gradually become water sensitive. At the end of the incubation period the parasites were washed 3 times with Elac and used for experiments, being denominated fresh schistosomula.

Incubation procedure

Fresh schistosomula (1,000/0.5 ml) were incubated in 10 ml vol screw-cap tubes for the time stated in results at 37°C in a humid atmosphere of 5% CO_2 in tissue culture medium NCTC-135 or Eagle Earle BSS (both from Difco Laboratories, Detroit, Mich. USA), containing 200 U/ml of penicillin and 200 µg/ml of streptomycin. For acquisition of protection a partially purified serum fraction (PPSF) from rabbit serum, eluted from Sephadex G-200 column (Tavares *et al.*, 1978) was added at a concentration of 200 µg protein/ml unless otherwise stated. Concanavalin A (Con A) (Worthington Biochemical Corp., Freehold, New Jersey) and α-methylmannoside, competitive inhibitor of Con A, were used in the culture medium at various concentrations and conditions detailed in the text.

Cytotoxic assay

Fresh or cultured schistosomula (1,000/tube) in various stages of development from 0-18 h were treated at room temperature for 30 min under sterile conditions with 0.1 ml of an appropriate dilution of heat-inactivated (56°C, 30 min) immune serum. After 30 min the parasites were washed with 1.0 ml of Elac and resuspended in 0.45 ml complex medium (Elac + 10% fetal calf serum) with 200 U/ml of penicillin and 200 µg/ml of streptomycin. After addition of 50 µl guinea-pig serum

(fresh or stored in liquid nitrogen) the schistosomula were incubated overnight at 37°C in a humid atmosphere of 5% CO_2. The next day, aliquots of 50-100 µl were counted in a stereomicroscope (Wild, Heerbrugg, Switzerland) and the percentage of living schistosomula was calculated. The living parasites were considered resistant to the lethal antibody. The control for complement (C) killing (alternative pathway) consisted of a group of tubes from which the parasites were not treated with immune serum. Only those experiments were considered in which consistent experimental conditions were revealed by a control with heat-inactivated guinea-pig serum (Ci). The percentage dead or damaged schistosomula of this control never exceeded 10%.

Incorporation of ^{14}C-labelled amino acid by the parasite

Fresh schistosomula were washed twice with M-109 (NCTC-109 modified by omitting the labelled amino acid, coenzymes, glucurono lactone, sodium glucuronate and tween 80) containing 200 U/ml of penicillin and 200 µg/ml of streptomycin. These schistosomula (40,000/vial) were incubated in 30 ml glass screw-cap vials in 2 ml of M-109 for 18h at 37°C, 5% CO_2, in the presence or absence of PPSF (200 µg protein/ml) or Con A (5 µg/ml), with 2 µCi ^{14}C-amino acid (NEN, 2 mCi/mg). At the end of the incubation period 0.05 ml (aliquots) containing about 1,000 parasites, were taken and tested in the cytotoxic assay. The remaining parasites were washed 3 times with 3.0 ml Earle's saline, pH 7.4, containing 0.01M cold amino acid (isotopic dilution) and used for tegument extraction.

Tegument extraction

The tegument of schistosomula was removed by addition of 2 ml 0.3M ice-cold $CaCl_2$ under continuous whirling on a Vortex Genie (position 8) during 7 min. After centrifugation at low speed for 30 sec the supernatant containing the labelled tegument was aspirated with a Pasteur pipet and conserved on ice. The schistosomula were washed 3 times with 1 ml 0.3M $CaCl_2$ whirled 1 min Vortex and the supernatants pooled. These supernatants containing the labelled tegument were either processed for radioactivity determination or used for gel electrophoresis fractionation.

Polyacrylamide slab gel electrophoresis

Lyophylized tegument preparations obtained from 200,000 to 400,000 schistosomula, incubated in the presence of labelled amino acid as described, were processed for polyacrylamide gel electrophoresis, as previously described (Cordeiro and Gazzinelli, 1979).

Radioactivity determinations

1. To the supernatants containing the tegument material 50% TCA was added to a final concentration of 10%. After 20-30 min on ice the solution was passed through a filter of glass-fiber paper (GF/C, 2.4 mn, Whatman) using a vacuum flask filter system (Pyrex, Millipore Corp.), the precipitate washed with 5% TCA and dried on the filter paper. Then the filters were processed for radioactivity determination in a liquid scintillator counter (Beckman LS-150) as described by Tavares *et al.* (1980). After solubilizing the protein from the filters according to Nagai *et al.* (1977) the protein was determined by the method of Lowry *et al.* (1951).

2. Gel slices 1 mm thick were cut and placed in a scintillation vial containing 0.5 ml of 30% H_2O_2, capped tightly, and incubated at 60°C for 12h. Following this means of dissolving the gel, 0.1 ml of 1% Triton X-100 (Scintillation Grade Amersham-Searle) and 10 ml of scintillation fluid (5 g of 2,5-diphenyloxalole, 0.5 g of 1,4-Bis [2-(5-feniloxazolyl] -benzeno and 100 g Naphthalene per liter of dioxane) were added. The samples were counted in a Beckman LS-150, model 1694. A counting efficiency of approximately 49% for tritium and 60% for ^{14}C was obtained for all sample slices. Quenching was monitored routinely by a channel ratio method. Differential quenching from vial to vial was not encountered. Spillover of ^{14}C counts into the tritium channel was subtracted from total counts in the tritium channel to yield net tritium counts. There was no spillover of tritium counts into the ^{14}C channel.

Indirect fluorescence assay

Fresh schistosomula or schistosomula incubated over-night in Eagle's medium in the presence or absence of either 200 µg of PPSF or 5 µg of

Con A were treated at room temperature with 250 µl of an appropriate dilution of human immune serum for 30 min. The serum excess was removed by washing the parasites 3 times with cold saline and then they were treated for 30 min at room temperature with a previously established dilution of a fluorescein goat anti-human globulin conjugate and washed 3 times. The intensity of fluorescence was scored in a fluorescence microscope (Olympus, Model E, Tokyo, Japan) on a scale from 0—4+. Before the schistosomula were fixed samples were taken for the cytotoxic assay.

Binding of ^{125}I-labelled IgG to schistosomula

The IgG fraction of human immune serum, obtained by gel filtration on Sephadex G-200, eluting buffer 0.05M NH_4HCO_3 + 0.02% sodium azide, pH 7.4 was lyophylized and resuspended in 1.5 ml Earle's saline pH 7.4. The fraction was iodinated by the Bolton Hunter Reagent ^{125}I (New England Nuclear) and dialyzed against Earle's saline, pH 7.4, until the radioactivtiy in the dialisate was 560 cpm (background level was 452 cpm) radioactivity was measured in a Beckman gamma 4,000 counting system. Schistosomula 1,000/tube), fresh or cultured in medium Eagle, Earle BSS for 18h at 37°C, 5% CO_2 in the presence or absence of 5 µg/ml Con A were treated with ^{125}I-IgG for 30 min at room temperature and washed 3 times with 0.5 ml Elac. Then the radioactivity of the schistosomula was measured. Schistosomula of each group were also tested in the cytotoxic assay as previously described.

Results

Only a low percentage of fresh schistosomula escape the lethal effect of the immune sera and complement. When they were first cultivated in a chemically defined medium, however, an increased percentage of resistant schistosomula appeared. Addition to the culture medium of 200 µg PPSF or 5 µg Con A resulted in a further increase in the number of parasites insensitive to the lethal antibody. Also, this occurred over a shorter period of incubation (Fig. 1). This effect was not due to an anticomplementary effect of PPSF or Con A since guinea-pig serum (source of complement) pre-incubated with either of these substances for 1h at room temperature, does not lose its cytotoxic activity. This

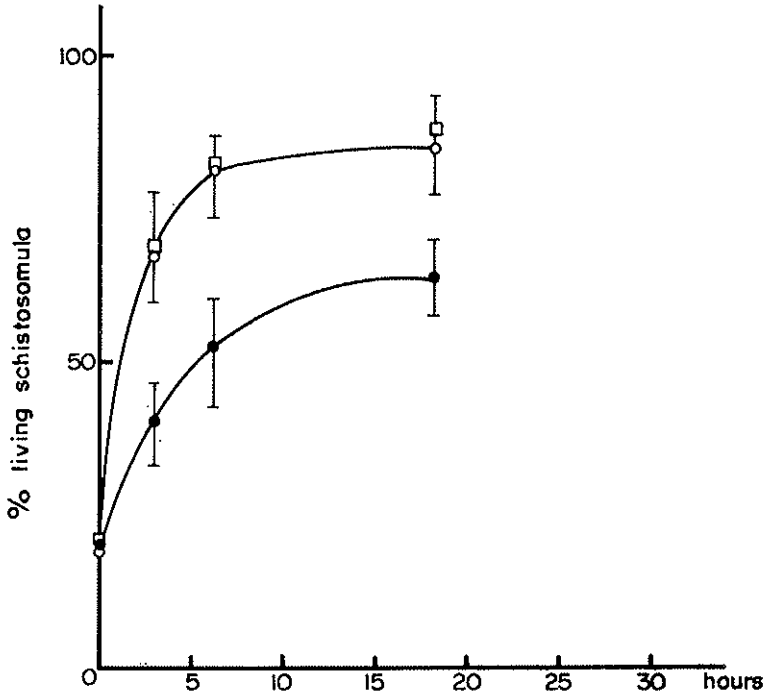

FIG. 1. Timing of development of resistance against antibody mediated killing by schistosomula incubated in a chemically defined medium in absence or presence of 200 μg of PPSF or 5 μg of Con A.

result was the same whether this pre-incubation was in the presence of antibody or in its absence.

In order to determine whether Con A and PPSF masked target antigens on the parasite surface, schistosomula pre-incubated with either Con A or PPSF were subsequently treated with human immune serum and fluorescein-labelled goat anti-human immunoglobulin. As shown in Table I no differences were revealed in the fluorescence of untreated and Con A or PPSF-treated schistosomula. Confirmation of the Con A results of the qualitative indirect fluorescence assay was obtained by using a ^{125}Iodine-labelled IgG fraction from human immune serum. No significant radioactivity differences were detected between the fresh schistosomula or schistosomula pre-incubated overnight with Con A (Table II).

As the death of the parasite probably occurs by a lesion on its

TABLE I — *Comparison of the anti-parasite antibody binding by fresh and cultured schistosomula.*

Schistosomula cultivated in:	Serum treatment	Intensity of fluorescence	Cytotoxic assay % living schistosomula ± S.D.
fresh	immune	+++	25 ± 5
fresh	normal	±	
NCTC-135	immune	+++	42 ± 7
NCTC-135	normal	±	
NCTC-135 + 200 µg PPSF	immune	++++	65 ± 7
NCTC-135 + 200 µg PPSF	normal	±	
NCTC-135 + 5 µg Con A	immune	+++	73 ± 5
NCTC-135 + 5 µg Con A	normal	±	

Samples of fresh and cultivated schistosomula were taken for indirect fluorescence and cytotoxic assay as described in Material and Methods.

TABLE II — *Uptake of radioiodinated IgG by fresh and cultured schistosomula.*

Schistosomula cultivated in:	Iodinated IgG bound cpm/schistosomula	Cytotoxic assay % living schistosomula ± S.D.
fresh	11.69	29 ± 5
NCTC-135 (18h)	14.60	55 ± 8
NCTC-135 + 5 µg Con A (18h)	14.01	90 ± 10

Samples of fresh and cultivated schistosomula were taken for radioactivity determination and cytotoxic assay as described.

tegument and assuming, based on the above evidence, that Con A or PPSF did not block the binding of the antibody to surface target antigens, attempts were made to establish a relationship between the insensitivity acquired by the schistosomula and any alteration in the tegument Con A or PPSF-induced. It was first studied whether PPSF and Con A stimulated protein synthesis in the schistosomular tegument during the period of acquisition of resistance by the organisms. As shown in Table III the incorporation of labelled amino acid into the proteins of the tegument was not stimulated by Con A while the presence of PPSF over the same period (18h) led to a significant increase in the specific activity of these proteins. In order to verify whether the serum induced any alteration in the tegumental proteins a double labelled experiment was done. Schistosomula incubated with ^{14}C-leucine in the presence of dialyzed serum for 18h were washed and reincubated with tritiated Leucine for an additional 10h. As depicted in fig. 2 the stimulation of protein synthesis by serum factor (s) does not result in a qualitative alteration of the protein pattern revealed by comparative SDS-gel electrophoresis of the labelled tegument.

Changes in the schistosomula tegument during the development of resistance to the lethal antibody was further examined by using Con A as an exploratory agent. The data in Table IV show that when α-methyl-mannoside a competitive agent was added to the incubation media together with Con A the Con A-induced protection was partially prevented, sug-

TABLE III — *Incorporation of ^{14}C-lysine in the proteins of schistosomula tegument.*

Schistosomula cultivated in:	10^{-3} cpm/mg protein from schistosomula tegument	Cytotoxic assay % living schistosomula
MEDIUM I	165	40
MEDIUM I + 5 µg Con A	190	60
MEDIUM I + 200 µg PPSF	269	64

Schistosomula were incubated for 18h in M-109 with 2 µCi ^{14}C-lysine in the presence of Con A or PPSF. After taking samples for the cytotoxic assay the tegument was removed and radioactivity measured.

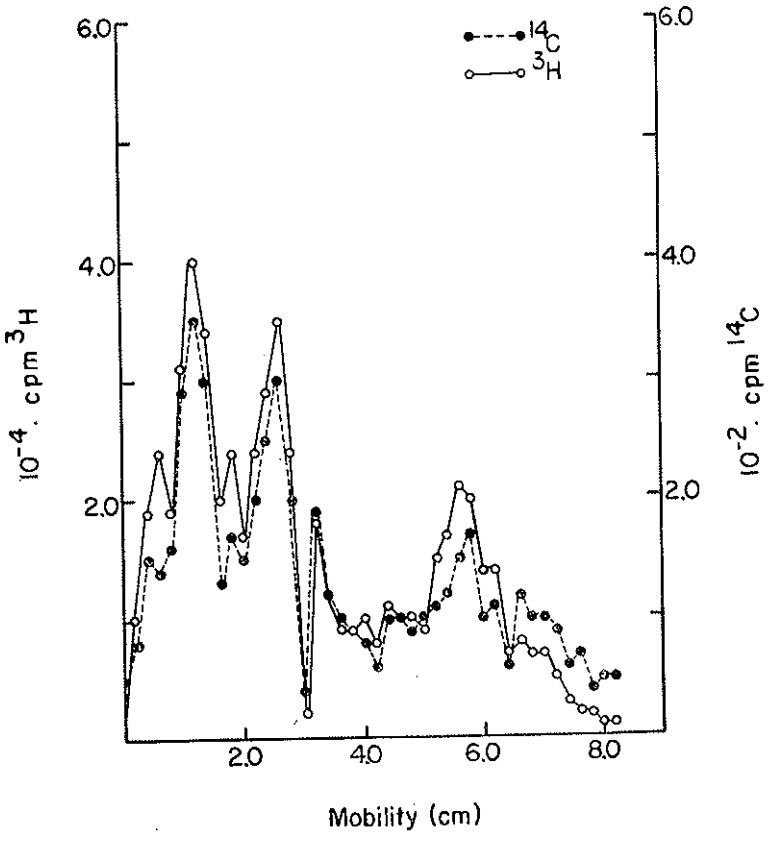

FIG. 2. Electrophoretic patterns of double labelled schistosomula tegument. Schistosomula incubated with ^{14}C-leucine for 18h were washed and re-incubated with ^{3}H-leucine at the same conditions. After 10h the tegument was removed and processed for electrophoresis and radioactivity determination.

gesting a specific binding at least for a significant part of Con A to the parasite surface. The Con A binding sites on the schistosomula surface seem to be gradually removed when schistosomula are treated with increasing amounts of trypsin. As shown in fig. 3 following trypsin treatment the schistosomula became more sensitive to the lethal antibody and the parasite capacity in developing resistance to the antibody-mediated killing in presence of Con A is lost. To verify the alterations produced in the schistosomula tegument following trypsin treatment radioiodinated schistosomula were treated with trypsin at the same conditions, their

TABLE IV — *Effect of α-methylmannoside on the protection induced by Con A in Schistosomula.*

Incubation medium	Cytotoxic assay % living schistosomula
NCTC-135	38
NCTC-135 + Con A	70
NCTC-135 + Con A + α−MM	56
NCTC-135 + Con A + α−MM	57
NCTC-135 + α−MM	34
NCTC-135 + α−MM	37

Con A 5 μg/ml
α-MM = α-methylmannoside 1.3×10^{-4} mmoles and 3.0×10^{-4} mmoles
Least significant difference by variance analysis for p = 0.05 : 9.05

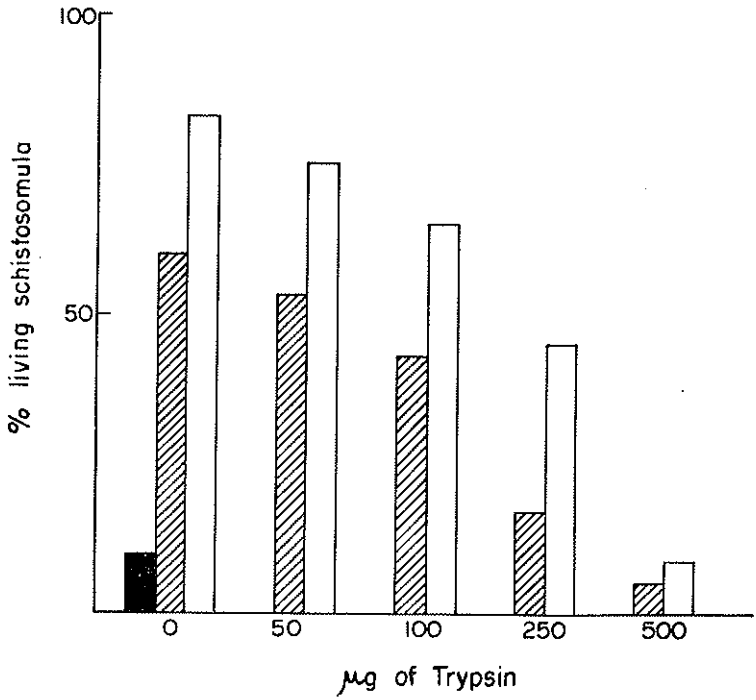

FIG. 3. Effect of trypsin treatment on the acquisition of protection by schistosomula against the lethal antibody. Schistosomula were pre-incubated with various concentrations of trypsin at 37°C for 30 min and then incubated overnight (1,000/ml) with (□) or without (▨) Con A. At the end of the incubation period cultured and fresh (■) schistosomula were submitted to the cytotoxic assay. In the controls the schistosomula death never exceeded 10%.

tegument extracted with 0.3M $CaCl_2$ and after solubilization it was run on 10% SDS-polyacrylamide gels electrophoresis under reducing conditions. Then gels were sliced and their radioactivity directly measured. The specific activity of the total proteins from the tegument dropped from about 7.1×10^6 to 5.2×10^6 dpm/mg of protein after trypsin treatment. As illustrated in Fig. 4 most of the high MW proteins or/and glycoprotein peaks are hydrolized by trypsin treatment. The increased radioactivity in the low MW region (6.5—8.0 cm) is probably due to the resulting polypeptide fragments. Further information about tegument changes in connection with the acquisition of resistance to the killing effect was obtained by adding Con A to the medium at different times during the incubation of schistosomula. The data in Table V show that the presence of Con A during a period of 5h from the beginning onwards is sufficient to induce maximum protection. In contrast, when Con A is present for a period of 13h but added after an initial incubation interval of 6 it results in only half of the maximum protection.

Discussion

As reported by Dean (1977) we found that schistosomula maintained in serum-free, chemically defined media become refractory to the effects of lethal antibody and complement. The rate of acquisition of protection as well as the number of resistant schistosomula could be increased signi-

Table V — *Acquisition of protection in relation to the stage of early development of schistosomula.*

Incubation time preceding Con A addition (h)	Time in presence of Con A (h)	Total incubation period (h)	Cytotoxic assay % living schistosomula
—	—	fresh	30 ± 5
none	18	18	75 ± 4
3	15	18	63 ± 5
6	12	18	52 ± 3

Schistosomula (1,000/ml) were incubated in Eagle's medium and at the times stated 5 μg/ml of Con A were added. After a total incubation period of 18h the cytotoxic assay was performed.

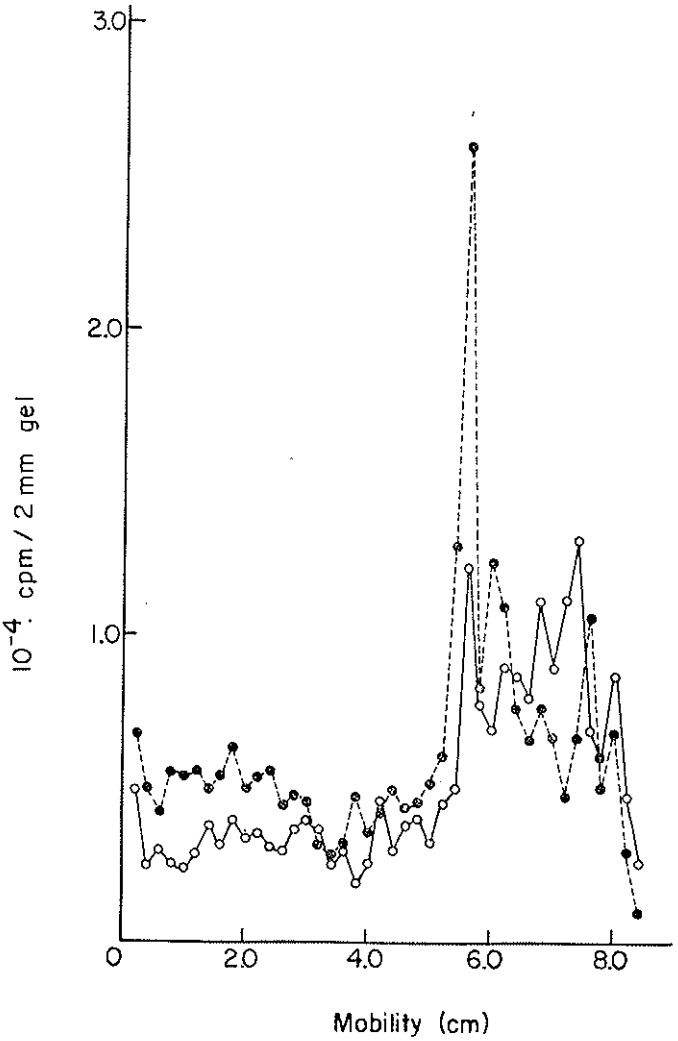

FIG. 4. Electrophoretic patterns of radioiodinated teguments before (o - - o) and after trypsin treatment (o — o). Two groups of 200,000 schistosomula were incubated with/without 500 μg of trypsin in 2.0 ml of NCTC-135 at 37°C. After 30 min the schistosomula were washed, iodinated and treated with $CaCl_2$ to remove their tegument. The tegument preparation was then processed for gel electrophoresis and radioactivity determination.

ficantly if PPSF or Con A are added to the media. These phenomena are more apparent when immune sera with high titers of lethal antibody are used in the cytotoxic assay (unpublished data). In the presence of PPSF or Con A this process begins after transformation and is completed in about 6h. During this period structural changes are known to occur in the parasite's tegument grown either "in vivo" or "in vitro". These changes include the development of a double unit membrane (Hockley and McLaren, 1973; Wilson and Barnes, 1977). It is unlikely, however, that this process by itself plays a key role in the resistance acquired against the lethal antibody because protein synthesis should be needed for the production of membrane components and Con A, which induced good protection, did not stimulate amino acid incorporation into the tegument (unless the membrane units are previously synthetized and stored as membranous bodies in subtegumental cells).

As an alternative mechanism it has been suggested that acquired host antigens would protect worms by masking susceptible parasite antigens on the surface membrane (Smithers, Terry & Hockley, 1969). This does not seem to be the case in the Con A- or PPSF-induced protection. Similar results have been reported by Moser *et al.* (1980). They found that the amount of anti-hapten antibody bound to TNP-conjugated skin and lung stage worms was comparable. On the other hand, evidences presented here suggest that young schistosomula seem to develop molecular changes in their tegument during cultivation "in vitro" which are related with the acquisition of resistance to the lethal antibody: 1) a progressive reduction of the protection induced by Con A as the time of culture before Con A addition increases may be interpreted as a diminishing number of Con A binding sites, since competitive inhibition by α-MM reduces the number of resistant parasites; 2) trypsin treatment which hydrolyzes high molecular weight components of the tegument increased the sensitivity of the schistosomula to the lethal effect of the antibody without altering the survival of the control organisms. At the same time the treated schistosomula lose their capacity to develop resistance when cultured in presence of Con A. Further experiments, however, will be needed to relate these phenomena since our data did not exclude the possibility that the trypsin treatment is removing besides Con A binding sites other glycoproteins which render the schistosomula more sensitive to the lethal antibody.

These changes if they occur are very subtle and are not revealed in the labelled tegument experiments.

It should be emphasized that our data suggest a relationship between the binding of Con A to specific sites on the surface of schistosomula and the rate of development of resistance by this parasite against antibody-mediated damage.

Finally, the data presented here clearly indicate that *S. mansoni* schistosomula gradually develop a resistance against the killing effect of immune sera *plus* complement. This occurs soon after their transformation and progress more quickly when Con A or serum factors are added to the culture medium. It has been previously shown that cultured schistosomula are also able to resist the attack by neutrophils (Novato-Silva *et al.*, 1980) and eosinophils (Dessein *et al.*, 1981) in presence of antibody and/or complement. In conjunction, these results suggest that the schistosomula are able to evade most of the immune effector mechanisms of the host, although *in vitro* studies do not allow definite conclusion as regards the *in vivo* situation. This possibility, therefore, introduces a further complication in the immunization against this parasite and should be taken into account in the studies attempting to induce a protective immunity in the host. In addition the comprehension of the phenomenon of the schistosomula resistance against the host immune response may open the possibility of developing drugs able to render the parasite more sensitive to the vertebrate defense mechanisms.

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STUDIES ON THE INDUCTION AND EXPRESSION OF RESISTANCE TO SCHISTOSOME INFECTION IN MICE

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We have been studying the resistance to *Schistosoma mansoni* infection which is induced in mice by a previous homologous infection or by immunization with gamma irradiated schistosome larvae. This presentation will provide a brief summary of our work on resistance induction and challenge infection elimination in these two models.

Our experiments on the resistance induced in mice by a previous *S. mansoni* infection have led to several conclusions. First, it appears that worms of both sexes are required for resistance induction. Unisexual infections of either sex did not induce detectable resistance under conditions in which bisexual infections induced nearly complete protection (Dean *et al.*, 1978a).

Second, most of the killing of challenge worms in bisexually infected mice appears to take place after the skin phase of migration, and probably after the lung phase of migration at earlier challenge times. In mice challenged 6 to 8 weeks after the initial infection, the recoveries of 6-day-old challenge worms from the lungs were not reduced in comparison with the recoveries from challenge control mice. In the same experiments, mice challenged at 12 or more weeks showed reductions of more than 50% (Dean *et al.*, 1978b). Preliminary experiments employing selenium-75

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labeled challenge cercariae indicate that most of the reduction in lung schistosomulum recovery observed at late challenge times can be attributed to events occurring after the arrival of schistosomula in the lungs. From the number of schistosomula detectable as spots on autoradiographs of the skin and lungs, it appears that in most mice showing large reductions in lung schistosomulum recovery, a small number of schistosomula are retained in the skin while most actually reach the lungs, but for some reason are not recovered. Tbese data provide some support for the report by Smithers and Gammage (1980) that skin schistosomulum recoveries are reduced at late challenge times.

Third, the process of challenge worm elimination appears to be associated with the pathological response to schistosome eggs trapped in host tissues. This conclusion is supported by (a) the inverse relationship between the number of schistosomula and number of eggs recovered from the lungs (Dean *et al.*, 1978b), (b) the protection of uninfected mice by the intravenous injection of eggs at a time ensuring simultaneous egg-induced granuloma formation and challenge worm arrival in the lungs (Dean *et al.*, 1978b), (c) the correlation among 10 strains of mice of resistance to reinfection with both portal hypertension and lung granuloma number (Dean, Bukowski and Cheever, 1981), and (d) the premature shunting of eggs into the lungs and reduction of lung schistomulum recovery by greater than 90% in 8-week-infected mice which had received ligatures of the hepatic portal vein (unpublished). The failure of portal ligatures to cause a reduction in the recovery of lung schistosomula from uninfected mice indicated that normal portal blood constituents shunted into the general circulation are not in themselves sufficient to prevent schistosomulum migration to or recovery from the lungs. The complete absence of resistance transfer from infected mice to their uninfected parabiotic partners, in spite of a continuous vascular exchange (Dean, Bukowski and Clark, 1981) provides indirect support for the idea that this form of resistance may depend on local processes, possibly occurring only in sites of egg accumulation.

The resistance induced by irradiated schistosomes, at least in mice, appears to differ fundamentally from that developing after a normal infection. While reinfection resistance appears to require patent adult infections, irradiated cercariae and schistosomula exposed to levels of gamma or X-irradiation whicb prevent their maturation have induced the highest levels of resistance. Also, irradiated 3- and 4-week-old liver worms induced

much lower levels of resistance than irradiated cercariae and lung schistosomula (Dean, Cioli and Bukowski, 1981).

The time and site of challenge worm elimination have not yet been identified in the irradiated larval model. Studies by Miller *et al.* (1980, 1981) indicated that cercarial challenges are eliminated primarily in the skin, and that lung-stage schistosomula introduced as a challenge by intravenous injection are not vulnerable to elimination. In contrast, our studies have provided several lines of evidence indicating that challenge elimination occurs largely or entirely after the skin phase, and probably after the lung phase, of migration. Lung schistosomulum recovery curves have consistently indicated that the peak recovery of lung schistosomula from immune mice is delayed several days in comparison with controls, but is reduced by an amount which accounts for no more than half of the reduction observed at the liver schistosomulum and adult worm stages (Minard *et al.*, 1978; Stek, Dean and Clark, 1981). These findings are supported by a recent experiment employing selenium-75 labeled challenge cercariae. The migration of labeled schistosomula from the skin to the lungs, and exit from the lungs, were delayed but not reduced in immunized mice. Histological studies of the lungs of immunized mice between 6 and 14 days after challenge infection revealed considerable augmentation of the normal inflammatory reaction around vessels containing schistosomula, but no evidence of damage to the worms (Clark, Dean and Stek, unpublished).

There are a number of variables, including assay systems and host and parasite strains, which need to be evaluated before we can explain the differences in results obtained with these resistance models hy different investigators. It would be even more premature to try to predict which observations would apply in man. Nevertheless, our studies thus far make two conclusions inescapable. First, the evidence of a link between pathology and resistance in reinfected mice greatly reduces our enthusiasm about the likelihood that the study of this model will provide us with a practical approach to vaccination. Second, our studies with irradiated cercarial immunization, as well as several recent reports of successful immunization of a variety of animals with irradiated schistosomes, indicate that this model is as likely as any currently under study to provide information which can eventually be applied in the development of a practical, highly effective vaccine.

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IMMUNE PROTECTION AGAINST *SCHISTOSOMA MANSONI* IN PERMISSIVE AND NONPERMISSIVE HOSTS

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The whole field of schistosome immunology appears to be at a turning point. Two major — and interconnected — events have significantly modified our current understanding of the most widely used experimental model, i.e. the mouse — *S. mansoni* system.

The mouse model

The first concept which is emerging is that resistance to reinfection as observed in the chronically infected mouse may be due — at least in part — to mechanisms which are only indirectly, or not at all, related with classical immune phenomena. Essentially, the failure of challenge infection worms to develop might be the result of some obstacle to their migration in a host which has been "modified" by the pathological consequences of the first infection. There are at least two ways in which this could happen: i) eggs which reach the lungs in late primary infection could be prejudicial to the subsequent migration of challenge schistosomula through the lungs [1]; ii) porto-caval shunts which are formed upon advanced liver disease could prevent post-lung worms to become established in the liver, which is the place where critical steps of schistosome maturation normally occur [2]. Several pieces of evidence concur to support this type of interpretation. Passive transfer of resistance is difficult to demonstrate when using mouse serum [3], a major obstacle against humoral immunity hypotheses. Single-sex infections, which cause no egg-related

pathology, do not induce resistance to reinfection [4, 5]. In a comparison of various mouse strains, the level of resistance to reinfection was found to correlate closely with the degree of portal blood pressure elevation and with the number of lung egg granulomas [6]. There is a temporal coincidence between the acquisition of resistance to reinfection and the development of collateral circulation from the hepatic portal vein to the vena cava [7]. Thus, there appears to be sufficient circumstantial evidence to suspect that chronically infected mice may not be a reliable model to study whatever immunological phenomena may be involved in resistance.

The second important development in experimental murine schistosomiasis consists in the demonstration that highly irradiated cercariae can induce in the mouse good levels of resistance against *S. mansoni* [8, 9], as in the case of rhesus monkeys infected with *S. japonicum* [10]. Since highly irradiated cercariae fail to complete migration and never reach the adult egg-producing stage, no major pathological changes are caused to the host. Also, there is evidence that, contrary to the resistance induced by a living infection, resistance induced by irradiated cercariae can be passively transferred in the mouse [6]. Thus, it appears that irradiated cercariae induce in the mouse a different type of resistance from that induced by normal cercariae. While the irradiated cercariae-mouse model is obviously a very attractive one, it is not yet clear whether similar immunization procedures would confer resistance in different host species. For instance, Taylor *et al.* [11] failed to induce resistance in baboons using irradiated *S. mansoni* cercariae and schistosomula. In my laboratory, unsuccessful attempts have been made to immunize Nile rats (*Arvicanthis niloticus*) with the same irradiated cercariae immunization which induced resistance in mice (Table I). However, it is possible that using different doses of irradiation, different doses of cercariae, different immunization schedules, the conditions may be found which will confer resistance to those host species in which no success has been achieved so far.

Nonpermissive hosts

In addition to the use of irradiated cercariae or schistosomula, another approach is presently available to study immune mechanisms in systems which are substantially free from pathological alterations. This is represented by the use of nonpermissive hosts, i.e. host species in which the schistosome infection is spontaneously blocked at some point during its development in the mammal. The laboratory rat is a typical example

TABLE I — *Resistance induced by irradiated cercariae.*

	Immunizing exposure		Challenge exposure	Worms recovered at 7 w (No. of animals)		%	Significance
	No. cercariae	Kr	No. cercariae	Control	Immunized	reduction	(P)
Mice	600	52	100	22.5 (12)	21.7 (12)	0.3	n.s.
Nile rats	600	52	150	70.8 (6)	81.3 (6)	—	n.s.
Mice	500	25	100	36.1 (12)	4.5 (18)	87.5	<0.001
Nile rats	900	25	100	52.8 (5)	49.7 (7)	5.9	n.s.

of a nonpermissive host for *S. mansoni.* In this species, after 4 weeks of essentially normal progression of the infection, worms stop further development and most of them die in the subsequent 2-4 weeks, while the survivors remain stunted and deposit only low numbers of non-viable eggs. Since a good resistance to reinfection can be easily obtained (and passively transferred) in the rat, this rodent appears quite suitable for a study of immune phenomena in the absence of gross pathological alterations. The spontaneous worm elimination, however, may introduce some complications, both in the practical calculation of worm recoveries and in the interpretation of results, since spontaneous elimination itself may be totally or partially an immune phenomenon. Therefore, I will first attempt to briefly review what is known about spontaneous elimination (sometimes also referred to as "self-cure phenomenon") and I will then proceed to examine the contribution of the rat system to the problems of resistance to reinfection.

Spontaneous worm elimination

The percentage of cercariae penetrating rat skin *in vivo* is roughly the same as with mouse or hamster skin [12]. This seems also to be the case *in vitro*, since rat skin is commonly used in many laboratories when preparing skin schistosomula [13], but strictly quantitative data are probably not easy to obtain due to the large variability of the *in vitro*

system. Early observations carried out 5-15 min after penetration, recorded a higher percentage of "dead" schistosomula in rat skin than in mouse or hamster skin (50% vs. 30% or 10%, respectively) [14]. However, more recent experiments carried out with an improved skin recovery technique, have resulted in roughly equivalent survival rates (about 35%) for rat and mouse at 24 hrs after infection [15]. Similarly, at the lung stage it has been shown that, when the appropriate techniques are used, recoveries as high as 39% of the input cercariae can be obtained at day 5, a yield which is at least as high as that obtained in mice [16]. Studies on the migration and the morphological development of schistosomula showed that, up to the 3rd-4th week after infection, all processes appear to proceed at a more or less parallel rate in rats and mice [17]. Scanning electron microscopy has revealed that a process of surface topography rearrangement which occurs in mouse worms between days 28 and 35 after infection, does not occur in rat worms until much later [18]. Schistosomes can be perfused from the rat liver as early as 6 days after infection and can be recovered in maximal numbers from this territory between the 3rd and the 4th week [19]. Peak recoveries can be as high as about 35% of infecting cercariae from young rats, but a clear host age dependence has been observed both in terms of worm yield and of peak recovery times [19]. Beginning at about week 4, worm recoveries show a steady decline, with a rather sharp drop between weeks 4 and 6 and a subsequent phase of slower worm elimination [20]. As the time progresses, the number of worms seems to approach asymptotically the baseline, but an average of about 5 worms could be recovered one year after infection with 1,000 cercariae [21]. In addition, the number of worms seems to reach a simewhat constant level late in the infection (e.g. 16 weeks) irrespective of the number of cercariae used for infection [21].

Mechanisms of spontaneous elimination

Discussing the possible mechanisms for the phenomena described above, Smithers and Terry [20] stated: "It is tempting to assume that the elimination of worms is due to an acquired immune response on the part of the host which becomes effective at the 4th week, but this has not been proved". In order to test this hypothesis, worm elimination was studied in immunosuppressed rats [22]. High levels of immunosuppression (both humoral and cellular) were achieved in animals which had been

thymectomized, irradiated and reconstituted with bone marrow cells obtained from thymectomized donors which were also carriers of a thoracic duct fistula. Immunosuppressed rats showed a significantly higher number of worms than intact controls at all times tested. However, spontaneous elimination eventually occurred in thymectomized rats as well, even though it was clearly delayed and less dramatic than in controls. In addition, worms recovered from immunosuppressed animals were as stunted as those from intact rats, females did not achieve maturation, egg deposition remained abortive and worm pairs failed to migrate from liver to mesenteric veins [22]. Thus, even though the immune system clearly plays an important role in worm elimination, immunological phenomena may be restricted to the final phases of killing and disposing of worms which have been initially "damaged" by some other non-immune mechanism.

Using a different approach, a series of experiments were designed to decide whether the timing of spontaneous rejection (from the 4th week onwards) was due to the fact that the rat needs just about 4 weeks to mount an immune response sufficient to kill schistosomes, or whether this timing was dictated by factors inherent to worm development. Schistosomes obtained from rats which had been infected 2, 3 or 4 weeks previously were transferred into the mesenteric veins of normal rats and the timing of worm elimination was determined in recipients [23]. It was found that 2-week-old worms were rejected 2 weeks after transfer, 3-week-old worms 1 week after transfer, and 4-week-old worms immediately after transfer (Fig. 1). Schistosomes obtained from mice or hamsters showed the same pattern of elimination in recipient rats. It was concluded that the onset of *S. mansoni* rejection in laboratory rats is dependent on the total age of the parasites and independent of the length of contact with the host in which rejection occurs. Thus, this kind of evidence can be taken as an additional argument against the hypothesis that spontaneous worm elimination is primarily an immune phenomenon.

We then attempted to gain additional information on the nature of the "inhibition" exerted by the rat upon *S. mansoni*, by asking whether rat schistosomes are subjected to some irreversible damage in this nonpermissive host or whether they are simply "held back" in a reversible way. The second alternative turned out to be the correct one, since schistosomes obtained from rats at any time after infection and transferred into permissive hosts, very rapidly developed into perfectly normal adult worms, migrated to mesenteric veins and started depositing the usual high num-

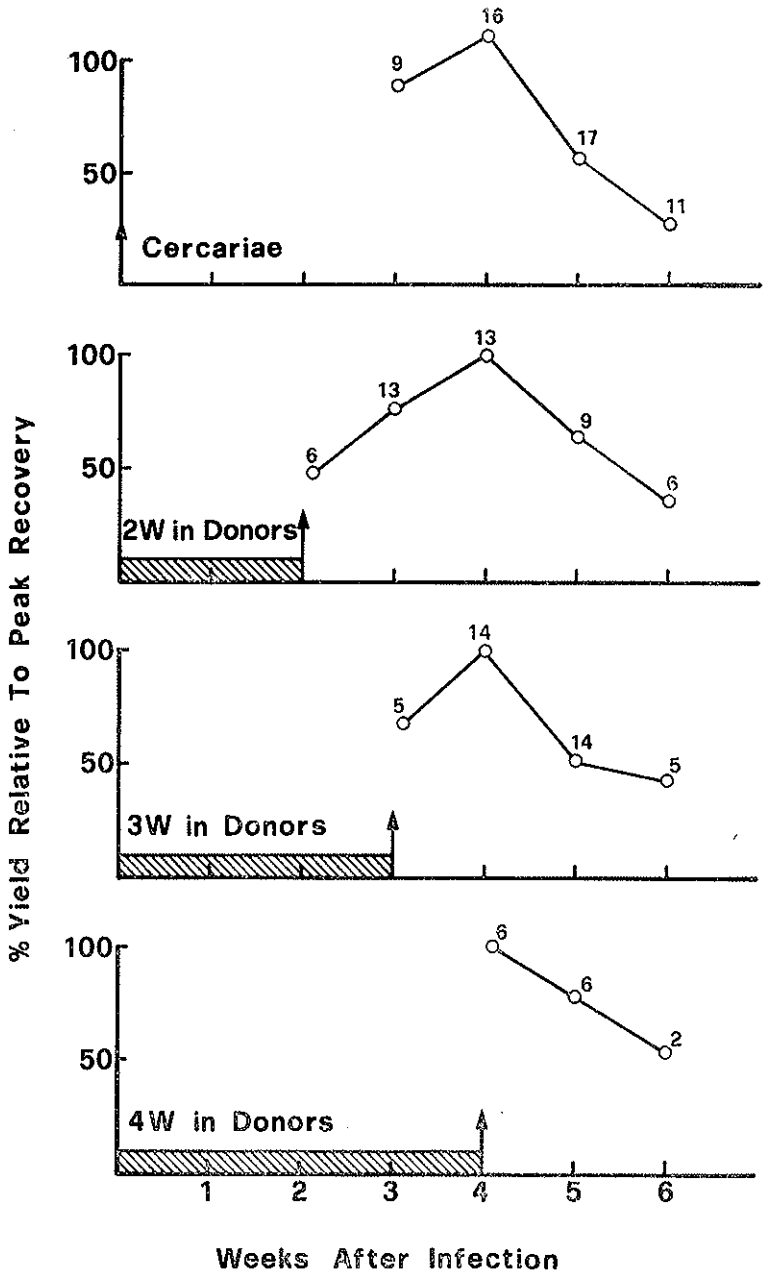

FIG. 1. Worm recovery from rats infected with cercariae or subjected to a transfer with rat worms of various ages. Worm yields are expressed as the percentage of maximum worm recovery obtained in each type of transfer. Each point is the average of three different experiments. The total number of rats employed is indicated next to each point.

bers of viable eggs [24]. Conversely, when fully developed fertile adult worms grown in a permissive host were transferred into the rat, their number was progressively reduced, the survivors failed to migrate to mesenteric veins, became stunted and were drastically inhibited in their rate of oviposition. These experiments indicate that the nonpermissive host does not only block worm development, but it also imposes severe restrictions upon the continuous maintenance of the adult state. The nature of these restrictions is not understood, but recent experiments by Knopf and Soliman [25] have raised the interesting hypothesis that schistosome development may be under the control of host hormones. In hypophysectomized or thyroidectomized rats onset of adult worm elimination was delayed, worm development was improved and oviposition increased.

Resistance to reinfection in the rat

Turning now to acquired resistance to schistosome infection in the rat, it is common experience to many laboratories that rates of protection between 60-80% can be routinely achieved in this host. Several parameters have an influence on acquired resistance and these will be briefly reviewed here. The number of cercariae in the primary infection should be above 10, significant protection being achieved with doses ranging from 30 to 5,000, with an optimum between 500 and 1,000 [26, 27]. Significant resistance can be observed already one week after primary infection, with maximal protection around the 4th-6th week [26]. This is an interesting feature of rat resistance, since the long time intervals which are needed in the mouse model are obviously a major factor in producing extensive cumulated pathological changes. Resistance is relatively long lasting in the rat, being maintained for about 40 weeks before any significant decline is observed [21]. When the challenge dose was increased from 1,600 to 5,000 cercariae, Knopf *et al.* [27] observed a drop in resistance at the higher dose, as if the protection potential could be somehow saturated. By varying the number of cercariae in both the primary and the challenge infection, the same authors convincingly demonstrated that resistance cannot be the result of an exhaustion of host nutritional capacities ("athrepsia") consequent to the increased worm burden, since resistance could be observed at final schistosome densities far below "saturating" doses. When the primary infection was killed by administration of antischistosomal drugs, resistance was reduced by about

50% if curing occurred during the first 4 days after infection, while there was an indication of a decreased resistance if drug cure was achieved at 6 weeks postinfection [21]. It should also be pointed out that in the rat, as in the mouse, there is evidence for strain related differences in the ability to develop resistance to reinfection [28]. A most critical factor in all experiments of resistance in the rat is the time interval between challenge infection and determination of worm burdens. Since spontaneous worm elimination would occur anyhow after week 4, perfusion must be performed at the time of maximum worm recovery, i.e. between 3 and 4 weeks after challenge infection. If worm numbers are determined later than week 4, the effects of spontaneous elimination are so dramatic that they soon overwhelm any resistance effect and thus render non-significant any difference between challenge controls and experimental animals [27].

Mechanisms of resistance

Having learned the lesson from the mouse model, one should then ask what is the evidence that resistance to reinfection in the rat is indeed an immune phenomenon. First of all, the same immunosuppressive maneuvers which, as described above, failed to abolish all the effects of spontaneous worm elimination, were completely effective in abolishing resistance to reinfection. Thus, thymectomized rats developed no protection following a primary infection which conferred resistance to intact control animals [28]. Thymectomy also largely abolished blood eosinophilia as well as tissue reactions to worms or eggs. Another important element in establishing that resistance is immunologically mediated, is represented by the successful passive transfer of resistance which could be achieved with either cells or serum from pre-infected rats. Non-adherent peritoneal cells were effective in transferring resistance when obtained from donors infected 3 weeks previously, whereas serum was maximally effective 7-8 weeks after infection [26]. It could be shown that serum protective activity resided in the Ig fraction, but serum transfer was effective only if performed not later than 3 days after challenge infection [26]. This indicates that the schistosome stages which are susceptible to immune killing are represented by the young pre-lung schistosomula. However, we also obtained evidence that if post-lung (2- or 3-week-old) worms were introduced into the mesenteric veins of pre-infected rats, a significant amount of resistance could still be shown to be effective against these schistosome stages (Table II). This may be an

TABLE II — *Resistance of pre-infected rats to schistosomes of 2 or 3 weeks of age.*

Challenge schistosomes			% worms recovered (No. of rats)			
No.	Age	Origin	Control	Pre-infected	% reduction	% resistance to cercarial challenge
200	2 w	rat	76.0 (5)	39.0 (4)	48.7 ***	51 **
200	3 w	rat	42.9 (5)	24.1 (6)	43.8 *	62 **
150	3 w	mouse	47.9 (5)	26.1 (5)	45.5 *	N.D.

Recipient rats were infected with 700-800 cercariae 8-11 weeks before challenge. Challenge organisms were administerd via mesenteric vein injection. Worm recovery was determined 2 weeks after challenge. Notice higher recoveries when total worm age at perfusion was 4 weeks instead of 5. Significance (P): * < 0.05 ** < 0.01 *** < 0.001

indication that multiple sites of rejection can, if necessary, be active in the rat. Resistance to cercarial challenge, on the other hand, can be induced by transplanting adult worms in the mesenteric veins of rats, an indication that the early developmental stages of the life cycle are not necessarily required to stimulate protection [29]. In the course of the above experiments it was also found that acquisition of resistance was always accompanied by elevated peripheral eosinophilia and humoral anti-schistosome antibodies. Manipulations of the rat which resulted in only one of these phenomena, failed to induce resistance. Irradiated cercariae (5 Kr) stimulated slightly less resistance than live cercariae and this resistance could also be passively transferred [26, 30]. Numerous *in vitro* tests have been developed in which rat cells, rat serum, or both, can be shown to produce adverse effects on newly transformed schistosomula. However, the possible relevance of such *in vitro* tests to the *in vivo* situation is not easily demonstrable, and in some cases it has in fact been excluded [31]. In any event, the availability of rat sera which are capable of passively transferring resistance, is of great potential interest in the study of schistosome antigens which elicit resistance and which are the target of such resistance.

In summary, then, useful information can be obtained by a comparison of various experimental models of schistosome immunity, and non-permissive hosts seem to offer some possible advantages for the study

of immune phenomena in systems which are essentially free from pathological complications.

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RECENT ADVANCES IN THE IMMUNOLOGY OF LEISHMANIAL INFECTIONS (WITH PARTICULAR REFERENCE TO THE PROBLEM OF VACCINATION)

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I. Introduction

The definition of effective immunoprophylactic measures in leishmanial infections remains a major challenge for immunologists. One problem encountered by those engaged in this field is that our understanding of the mechanisms of immune defence against *Leishmania* parasites is far from adequate. Thus, the respective roles of humoral and cellular immunity in protection against *Leishmania* have not yet been fully assessed. In addition, by virtue of its intra-macrophage location, the parasite does not appear to be readily accessible to some of the usual effector mechanisms of immunity. In this short review, some of the main points relevant to the problem of host protection will be briefly discussed, before considerations more specifically directed to the question of vaccination are presented.

I.A *Characteristic features of* Leishmania *parasites*

Leishmanias are protozoan parasites capable of infecting a variety of vertebrates, including man. From an immunological standpoint, one of the most significant features of *Leishmania* parasites is that, in mammalian hosts at least, they are obligatory intracellular: they live and multiply within cells of the mononuclear phagocyte system. This means that during

the course of evolution, these microorganisms have developed protective devices that enable them to survive in the very cells that are responsible for the elimination of undesirable intruders.

In their intracellular location, *Leishmania* parasites take the form of round or ovoid bodies 3-4 μ in length, known as *amastigotes* ("without a flagellum"). *Leishmanias* are transmitted hy the hite of sandflies. The vector becomes infected by feeding on a diseased host: parasitized macrophages are taken up, they release parasites which multiply in the alimentary tract of the insect, move to the proboscis, and are expelled when the sandfly bites again. In their vector form, the parasites are flagellate and motile *promastigotes*, which can also be maintained in culture [21].

A considerahle number of leishmanial species have been described, that produce in infected hosts various types of pathological symptoms and immune responses. Based on the experimental observation that the form of infection (e.g. healing vs. non-healing) produced by inoculation of *Leishmania donovani* [10, 11] or *Leishmania tropica* [5, 35, 59] to mice shows considerable variations, depending on the genetic background of the host, it is generally accepted that the clinical features of leishmanial infections result from the interplay between genetic factors contributed by both the host and the parasite. In the mouse, the importance of factors controlled by genes within and without the major histocompatibility complex has been stressed [8, 27]; in man, a possible role for the ABO blood group status in determining susceptibility to *Leishmania* has been similarly postulated [33].

I.B *Disease forms produced by* Leishmania *infections in man, and their immunological characteristics*

I.B.1 Cutaneous leishmaniasis.

Cutaneous infections in the Old World (the *Oriental sore*) and in the New World are caused essentially by parasites that belong to the *L. tropica* and *Leishmania mexicana* complexes of species, respectively. The typical cutaneous ulcer remains usually confined to the skin around the infecting bite, and heals (usually) spontaneously within several months; people are then considered to be permanently immune. The main immunological characteristics of this condition are both a marked cell-mediated reactivity to parasite antigens, which is manifested by a strong response in the delayed skin test [28] and relatively low levels of antibodies [13]. Peripheral

lymphocytes from patients and recovered individuals also undergo blast transformation *in vitro* in presence of extracts of *L. tropica* [69]. A remarkable feature of these cellular reactions is that they become positive well *before* protective immunity or healing is evident. A similar situation has been described in the experimental cutaneous infection of the guinea-pig by *Leishmania enriettii* [15].

In addition to the classical *Oriental sore*, other forms of cutaneous leishmaniasis can be induced by infection with *L. tropica*. It has been proposed that these different clinical entities distribute along a spectrum of immunological and histological characteristics [26]. Two markedly distinct "aberrant" forms of the disease, namely *leishmaniasis recidiva* and *diffuse cutaneous leishmaniasis*, would lie at opposite ends of this spectrum. In *leishmaniasis recidiva*, a previously healed ulcer becomes reactivated; the lesion consists predominantly of a lymphocytic infiltrate and of rare histiocytic elements. Parasites are very scanty, and often remain undetectable. Cellular immunity (as assayed in the delayed skin test) is extremely marked [63, 66]; the reason for failure to heal in spite of this high reactivity is unknown. *Diffuse cutaneous leishmaniasis* (DCL) constitutes the other pole of this spectrum. This condition is characterized by the appearance of numerous papular or nodular eruptions, resulting from parasite spreading through the lymphatics or by wandering macrophages. The infection remains dermatotropic, however, and no ulceration can be observed; the lesions consist of histiocytomas containing an abundance of parasites. The main immunological feature of this condition is that *patients with DCL do not respond in the delayed skin test* [14]. The last aberrant form of cutaneous leishmaniasis is the disease known as *post-Kala-azar dermal leishmaniasis* (PKADL), which corresponds to a cutaneous relapse of cured visceral leishmaniasis caused by *L. donovani*. Acquisition by the parasite of a dermatotropic behavior is unexplained Striking similarities exist between DCL and PKADL at the clinical and histological levels, suggesting a common mechanism of induction.

I.B.2 Mucocutaneous leishmaniasis

Subspecies of the *Leishmania braziliensis* complex are responsible for this clinical form, which occurs mostly in South America where it is known as *Espundia*. Mucocutaneous leishmaniasis resembles a simple cutaneous ulcer in its initial stages; through metastatic spread, the parasite establishes itself in the mucous membranes of the nose, mouth, pharynx and larynx.

Necrosis and erosion of the infected tissues then follows, with perforation of the palate and loss of the nasal septum, a cause of major disfigurement. The disease usually runs a fatal course, death occurring through secondary infections such as bronchopneumonia. Espundia patients respond well to *Leishmania* antigens in the delayed skin test, and antibodies are detectable by immunofluorescence [22, 67]. It is conceivable that failure to heal be due to the anatomical location of the parasite, which might preclude the normal functioning of the effector arm of immunity.

I.B.3 Visceral leishmaniasis

Invasion by *Leishmania* parasites of the *L. donovani* complex, of the reticular syncytium of internal organs, particularly the spleen, liver, bone marrow and lymph nodes, gives rise to the disease known as *Kala-azar*. This condition is usually fatal if left untreated. The clinical picture is characterised by anemia, leukopenia, amyloidosis, and by a reversal of the normal albumin/globulin ratio, due to a considerable level of hypergammaglobulinemia, particularly of the IgG and IgM immunoglobulin fractions [31, 61]. Part of the newly formed globulins consists of antibodies to *Leishmania* antigens. It is usually accepted that *the delayed skin test is negative* during tbe active phase of Kala-Azar of the Mediterranean basin, India and South-America [47, 65]. Skin hypersensitivity becomes positive after cure, whether spontaneous or drug-induced. Similarly, the *in vitro* lymphoproliferative response to *Leishmania* antigens of peripheral blood leukocytes from Kala-azar patients remains undetectable before cure; following a successful course of therapy, it turns positive as does the skin test [71].

II. The role of the macrophage in Leishmanial infections

As mentioned above, *Leishmania* parasites are obligatory intracellular microorganisms which live in cells of the mononuclear phagocyte system. *Leishmanias* do not seem to actively penetrate into their host-cells; rather, they are engulfed by a normal phagocytic process. Internalization requires a preliminary attachment of the parasites to the macrophage membrane, presumably through interaction of lectin-like structures on the phagocytes [40] and appropriate ligands [25] on the microorganism, or vice-versa. In their intracellular location, the parasites are enclosed in a

vacuole, whose membrane is derived from the plasma membrane of the macrophage. Ultrastructural investigations have shown that secondary lysosomes fuse with the parasitophorous vacuole [3, 7, 20], thus presumably exposing the parasite to the lysosomal contents. In addition, phagocytosis of *L. donovani* and of *L. tropica* promastigotes by mouse macrophages *in vitro* has been shown to be accompanied by a burst of oxidative metabolism in the phagocyte, which leads to killing of most of the microorganisms [52]. Thus, in their promastigote form at least, *Leishmania* parasites do not seem to be well adapted to intracellular living. However, the few organisms that may be able to survive the initial assaults of the phagocyte's microbicical machinery, will transform to *amastigotes*, a developmental stage apparently more fit for intracellular survival. In this connection, it is perhaps significant that promastigotes of *L. donovani* have been observed to infect human skin fibroblasts *in vitro* [19], in which cells transformation to amastigotes takes place in the absence of phagosome-lysosome fusion, that is, in an environment presumably less detrimental to the parasite.

II.B *Macrophages as antigen-presenting cells*

Induction of an immune response requires that the immunogen be first processed by accessory cells, then presented by such cells to the lymphoid system in an appropriate context of surface structures coded for by genes within the major histocompatibility complex. When the immunogen consists of an intracellular parasite, the question can be raised as to what mechanisms allow antigens of the microorganism to reach and stimulate the immune system. In this connection, it is interesting to note that human and murine macrophages infected by *L. tropica* and *L. donovani* have been shown to display parasite-derived antigens on their surface [6, 29, 35]. Whether such antigens are the ones responsible for induction of the anti-*Leishmania* immune response is unknown, however.

Two points of relevance to the present discussion, as well as to the problem of vaccination in leishmanial infections, can be made. First, recognition of macrophage-borne parasite antigens by lymphocytes would be expected to constitute an important step not only in the *induction* of the immune response, but also in the *effector phase* of such response, particularly in the context of macrophage activation (Section II.C). In this respect, it is noteworthy that macrophage activation could be obtained by incubation of parasitized phagocytes *in vitro* in the presence of *Leishmania*-

immune lymphocytes from the spleen or lymph nodes from infected or immunized animals [45, 50], under experimental conditions excluding the presence of extracellular parasites. In this experimental model, activation is thought to result from the interaction between specifically sensitized lymphocytes and parasite antigen displayed on the surface of infected cells.

Second, indicated above (Section I.A), a considerable degree of variation can be observed in the response of different inbred strains of mice to inoculation with *L. tropica* and *L. donovani*. Thus, mouse strains can be classified as healers (or resistant), respectively non-healer (or susceptible), depending on the outcome of infection. Based on an indirect assay of the amount of histocompatibility structures on the surface of macrophages, it has been suggested that failure to heal in Balb/c animals (a mouse strain highly susceptible to infection by *L. tropica*) might be linked to a decreased expression of H-2 antigens by *infected* macrophages of that particular strain, as compared to non-infected macrophages, or to infected macrophages of a resistant strain [35]. Infected macrophages of susceptible strains would thus be inferior in their capacity to stimulate an anti-leishmanial immune response. Considering the requirements for adequate histocompatibility matching between lymphocytes and macrophages in the process of macrophage activation (cf. Section II.C.), decreased H-2 antigen expression by the latter cells might similarly lead to a reduced capacity for activation and for intracellular parasite killing. If correct, these observations would suggest that failure to heal is linked to a parasite-induced defect at the level of the macrophage-lymphocyte cooperation. It is uncertain whether such a defect could be reversed by vaccination.

II.C *Macrophage activation as an effector mechanism of immunity in leishmanial infections*

II.C.1 General characteristics of the activation process

Macrophage activation is defined as a process whereby the phagocytes acquire an enhanced capacity to phagocytize, kill and digest microorganisms. At the biochemical level, activation is characterized by the production of a wave of metabolites of oxygen that are toxic for both intracellular pathogens [18, 53] and for other, extracellular targets [55]. These metabolites appear to include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxyl radical ($HO^\cdot$); their mode of synthesis is presumably similar to that observed in phagocytizing

cells [39]. Activation would thus serve to restimulate those metabolic processes required for the killing of intracellular microorganisms, whenever pathogens have been able to survive the toxic mechanisms normally evoked during the phagocytic act.

Various stimuli can induce in macrophages the morphological, biochemical and functional changes characteristic of the activated state [reviewed in ref. 48]. Of particular interest to the question of resistance against leishamanial infections is the finding that macrophage activation constitutes a normal effector phase of cellular immune responses. As originally described by Mackaness and several other investigators in the model infection of mice by *Listeria monocytogenes*, activation is mediated by immune lymphocytes of the T lineage, which become stimulated upon interaction with the specific antigen [43, 46]. This results in the elaboration of soluble factors (lymphokines, interleukines) endowed with macrophage activating properties [17].

Activation of macrophages is thought to constitute an essential mechanism of immune defense against a number of intracellular microorganisms and, perhaps against metazoan parasites as well [48]. In particular, it has been proposed that healing of leishmanial infections critically depends on the capacity of infected individuals, or animals, to activate their macrophages adequately [49]. The evidence pertaining to this point is briefly reviewed below.

II.C.2 Role of cell-mediated immune reactions in resistance against leishmanial infections

It is usually accepted that immune resistance against leishmanial infections depends on the cellular (as opposed to the humoral) component of the immune response. This notion is derived from clinical observations as well as from investigations of experimental laboratory models of leishmaniasis. Thus, the self-healing forms of cutaneous infections in man are accompanied by strong hypersensitivity reactions of the delayed type to intradermal inoculation of parasite antigens, whereas antibodies can be found only in low titers [reviewed in ref. 49, 66]. Conversely, *non-healing* leishmanial infections (visceral leishmaniasis, diffuse cutaneous leishmaniasis) are characterized from an immunological standpoint by an absence of cellular reactions, whereas antibodies are present. Moreover, in the case of Kala-azar, delayed skin hypersensitivity reappears after cure and corresponds to a state of protection against reinfection. Although a

correlation can thus be established between healing and the occurrence of cell-mediated immune reactions, it has to be stressed that situations have been reported in which cellular reactions do not seem to benefit the host, as in the *recidiva* form of leishmaniasis where lesions fail to heal in spite of the capacity of the host to develop high reactivity of the delayed type to the parasite or its products.

A situation similar to that outlined above has been described in the study of experimental *Leishmania* infections of laboratory animals. Intracutaneous inoculation of guinea-pigs with *L. enriettii* produces a nodule, that ulcerates after several weeks, then heals leaving the animal permanently immune against challenge infection [15]. An excellent level of cell-mediated immunity is demonstrable *in vivo* (by the delayed hypersensitivity reaction), as well as by *in vitro* techniques such as blast transformation or inhibition of macrophage migration [9]. Procedures aimed at decreasing cellular reactivity, such as whole-body X-irradiation and induction of tolerance by intrafoetal injection of soluble parasite antigen, may enhance considerably the severity of the lesions [16]. Finally, induction of delayed hypersensitivity reactions to *unrelated* antigens at the site of parasite inoculation may be sufficient to block totally the development of leishmanial nodules [4].

More striking still are findings described in the study of experimental infections of mice by *L. tropica* and *L. donovani*. Evidence that recovery is mediated by cellular rather than humoral mechanisms can be summarized as follows: *a*) studies with Biozzi's "high" and "low" responder mice indicate that resolution of cutaneous *L. tropica*-induced lesions is independent of the level of humoral antihody [34]; *b*) cell-transfer experiments clearly point to the role of lymphocytes of the T lineage in mediating protection against both types of microorganisms [60, 62]; *c*) in animals that belong to strains (e.g. Balb/c) categorized as genetically "susceptible" to *L. tropica* infection, failure to heal is accompanied by decreased reactivity of the delayed type at the skin level [37]. Conversely, animals of the susceptible genotype that are immunomodulated in an appropriate fashion (as by sub-lethal X-irradiation) may regain both the capacity to produce delayed reactions upon intradermal inoculation of leishmanial antigen, and the faculty to heal skin lesions induced by injection of the parasite [38] (cf. Section III.C).

II.C.3 Role of the activation of host-macrophages in the destruction of intracellular *Leishmania* parasites

Although the precise mechanisms by which the cellular reactions developing in the recovering host mediate intracellular parasite destruction have not been established, the available evidence suggests that immune induction of *macrophage activation* plays a crucial role in this process. Non-specific stimulation of the mononuclear phagocyte system by BCG [68] and glucan [24] has been shown to considerably increase the resistance of meci against infection by *L. tropica* and *L. donovani*, respectively. However, the best evidence of a role for activated macrophages as mediators of recovery come from *in vitro* studies of the function of such cells.

Thus, it has been shown that murine macrophages activated in a variety of non-specific ways acquire the capacity to destroy *in vitro L. enriettii* and *L. tropica* [5, 17]. Moreover, a parallel could be established between the capacity of *in vitro*-activated murine macrophages to destroy *L. tropica*, and the origin of such macrophages: activated macrophages from "resistant" mouse strains were far more efficient at killing and digesting the parasite than were activated macrophages from susceptible animals. Based on these observations, a hypothesis has been formulated, according to which failure to heal *L. tropica* infection in susceptible strains might be attributed to a genetic deficiency of activated macrophages from such strains to eliminate the parasite [5].

That macrophage activation resulting from a cell-mediated response against parasite antigens, may be an effector mechanism of protection against leishmanial infections in the recovering host, is further supported by the demonstration that *specific* activation can be obtained *in vitro* by incubation of parasitized macrophages with lymphocytes from lymph nodes and spleen from recovered or immunized animals [45, 50]. Such activation may lead to destruction of the intracellular microorganisms: it is specific, as shown by the fact that incubation of parasitized macrophages with lymphocytes sensitized against another antigen, such as ovalbumin, does not induce activation of parasitized macrophages. As discussed above, macrophage activation under such experimental conditions is thought to result from the stimulation of immune lymphocytes upon interaction with parasite antigens borne on the macrophage membrane.

III. Mechanisms of parasite survival in the genetically susceptible host

As described above, non-healing forms of leishmaniasis occur both naturally in man, and experimentally in laboratory animals. In man, chronic leishmaniasis has been associated with a state of anergy at the cell-mediated level of the immune response, as in DCL or Kala-azar as well as with hyperergy of such response, as in *leishmaniasis recidiva*. It is not clear, however, whether failure to heal represents the cause or the consequence of these immunologically deviant conditions.

An understanding of the mechanisms of parasite survival in otherwise immunocompetent hosts is essential to the design of appropriate vaccination procedures. Indeed, the nature of the deficiency in non-healer hosts may have a profound bearing on the type of vaccination to be used. Analysis of the non-healing mechanisms in experimental *Leishmania* infection of animals suggests at least three mechanisms whereby the host may fail to destroy invading leishmanias. The evidence for each of them is briefly outlined below.

III.A *Parasite survival in defective macrophages*

As discussed above, mouse strains can be classified as "healer" or "non-healer", depending on the outcome of infection by *L. tropica*. Animals of the latter category tend to maintain skin lesions for their lifetime, with a degree of visceral involvement in some strains, whereas the former heal their ulcer within a few weeks.

In a study of intracellular parasite destruction in *in vitro*-activated macrophages, it was observed that whereas activated macrophages from both healer and non-healer background destroyed intracellular *L. enriettii* (a parasite to which the mouse is not susceptible) with a similar degree of efficacy, only activated macrophages from *healer* background could eliminate *L. tropica* [5]. According to this model, failure to heal *L. tropica* infection in *non-healer* animals would thus result from some form of deficiency of the anti-leishmanial mechanisms of activated macrophages from such animals; conversely, *L. tropica* might be particularly well equipped to resist the anti-microbial processes of non-healer macrophages. The nature of the macrophage defect, or of the parasite survival mechanisms is unknown. Of relevance to this point is the recent demonstra-

tion that *L. tropica* as well as *L. donovani*, when grown extracellularly, are poorly endowed in hydrogen-peroxide-metabolizing enzymes such as catalase and glutathion peroxidase, and are thus particularly susceptible to lysis by oxidative mechanisms, in their promastigote form at least [52]. Whether amastigotes have a higher supply of such enzymes, and/or whether macrophages from non-healer animals are weaker in their capacity to generate toxic metabolites of oxygen upon activation compared to macropbages from healer strains, remains to be established.

III.B *Defective antigen presentation by parasitized macrophages from non-healer strains*

Interesting observations have been reported in the study of antigen presentation by *Leishmania*-infected macrophages. *L. tropica*-parasitized macrophages from non-healer (Balb/c) background (as opposed to healer macrophages) were reported to be unable to sensitize syngeneic recipients against *Leishmania* antigen, as measured in a delayed hypersensitivity assay [35]. This was found to correlate with an apparent loss of histocompatibility structures coded for by the K and/or D regions of the (H-2) major histocompatibility locus. Although the significance of these observations is uncertain, it is conceivable that infection of non-healer macrophages by *L. tropica* might induce a decreased expression of surface histocompatibility structures essential to the process of antigen presentation. Furthermore, if parasitized macrophages from non-healer background are characterized by a lower expression of Ia antigens, this would lead also to a decreased capacity of such macrophages to become activated by syngeneic effector lymphocytes, as the interaction leading to activation has been shown to depend on compatibility at the Ia level between both types of cells.

III.C *Generation of suppressor cells*

The non-healing phenomenon in mice infected by *L. tropica* has been analyzed from a third angle. As shown by Howard and collaborators, DTH reactions to parasite antigen in infected Balb/c mice are found to decrease as infection progresses [37]. Based on the demonstration that spleen cells of mice infected for 7 weeks contain a lymphocyte population that, when transferred to syngeneic recipients, decreases the capacity of

inoculated parasite antigen to sensitize these animals for DTH, it has been postulated that antigen-specific suppressor cells are generated during *L. tropica* infection. Moreover, low-dose irradiation prior to infection has been shown to induce healing in animals of susceptible genotype [38], an observation attributed to the selective destruction of antigen-specific suppressor cells in the irradiated animals.

IV. Vaccination against Leishmania parasites

Vaccination of man or laboratory animals against *Leishmania* parasites has been attempted by three different procedures: *a*) by the use of virulent, homologous organisms; *b*) by inoculation of "attenuated" organisms or extracts thereof; *c*) by injection of heterologous (that is, belonging to a different species), non pathogenic organisms. The rationale and results obtained by these three approaches are briefly discussed below.

IV.A Vaccination against Leishmania parasites

It is evident that the use of virulent organisms as vaccinating agents is applicable solely under circumstances where the infection thus produced is self-curable. Moreover, such procedure will be justified only when the benefits of future immunity largely outweigh the immediate inconveniences created by the disease. Thus, injection of virulent *L. tropica* in unconspicuous sites as a preventive measure against the cosmetic sequelae of cutaneous leishmaniasis, has been in empirical use for generations in Middle-Eastern countries, and is still commonly practised in Israel and the USSR [72]. The deliberately created lesions can then be followed under medical supervision. One of the essential advantages of vaccination against cutaneous Leishmaniasis is that only one sore is created at a chosen site, vs. several sores which often result from the natural, fly-borne infection [54]. Loss of virulence of parasite stocks is a commonly encountered problem [41], and recent developments are aimed at the production of stable, storable parasite preparations (e.g. frozen promastigotes) [32].

Animal experiments have been performed to determine whether reduction of the infecting dose to a level inducing only a subclinical infection, would still lead to protection against a challenge inoculum. In mice and hamsters, inoculation of subthreshold numbers of *L. tropica*

led to a degree of immune resistance against low challenge doses [59, 64]. Full immunity is apparently achieved only when a true infection is produced, however that is permitted to run its course and heal spontaneously. Extension of these results to other experimental systems is awaited with interest. Particularly relevant would be experiments aimed at protecting animals of high susceptibility to the disease, to mimic more adequately the conditions found in the natural infection of man by highly pathogenic leishmanial species such as *L. donovani*.

IV.B *Protection induced by "attenuated" organisms or parasite extracts*

Experiments with guinea-pigs indicated that irradiation of *L. enriettii* with high doses (> 25,000 R) of gamma rays abolished their vaccinating potential at the same time as it suppressed their capacity to produce an infection [44]. In one report, however, it was demonstrated that inoculation of hamsters with irradiated promastigotes of *L. donovani* appeared to confer protection when the animals were tested *late* (up to 130 days) after vaccination [23]. These results are of great interest, in view of the considerable susceptibility of hamsters to this infectious agent. Induction of protective immunity by parasite extracts has been attempted in a limited number of animal studies: some resistance to the cutaneous disease could be demonstrated following vaccination with sonicated parasites or a ribosomal fraction thereof [58, 60]. Finally, it has been shown that inoculation of man with killed promastigotes responsible for American dermal leishmaniasis induced a skin test response to *Leishmania* antigens detectable for up to three years in a proportion of the vaccinated group [51]. No information was presented, however, regarding actual protective immunity in such individuals against the naturally occurring disease.

Interesting observations have been reported in the study of vaccination of man against Kala-azar. Epidemiological surveys of populations living in areas of East-Africa endemic for visceral leishmaniasis have shown that the proportion of healthy individuals displaying a positive leishmanin reaction (1) increases with the duration of residence in these areas [30, 47]. This observation suggested that a positive skin test

(1) The test measures the delayed hypersensitivity reaction induced by inoculation in the skin of dead parasites or extracts thereof.

could be conferred by exposure to *Leishmania* parasites from wild reservoirs, that would induce subclinical infections only. Trials on a group of 119 volunteers indeed demonstrated that inoculation of a strain of *L. donovani* isolated from a wild squirrel would produce a mild skin infection only, and would convert a negative leishmanin reaction to a positive one in a large proportion of the vaccinated individuals [47]. When skin-positive reactors were challenged with a human strain of *L. donovani*, no leishmaniomata or Kala-azar resulted during the subsequent 18 months. When a larger segment of the population was immunized with the rodent parasite, however, no protection against the natural infection appeared to have been induced [36]. It is thought that the vaccinating organism might have lost immunogenicity after prolonged maintenance in the laboratory.

IV.C *Vaccination by heterologous, non-pathogenic organisms*

The rationale for attempting to use heterologous, non-pathogenic organisms as vaccinating agents against the more virulent parasites is based on the remarkable level of immunological cross-reactivity between leishmanial species at both the humoral and cellular levels. Thus, *L. mexicana*-infected individuals display a positive skin response to *L. tropica* as well as to *L. mexicana* [1]; patients recovered from East-African Kala-azar similarly react against *L. braziliensis*, *L. tropica* and *L. enriettii* [47]. Cross-hypersensitivity at the skin level can be demonstrated in guinea-pigs between antigens from *L. enriettii*, *L. donovani*, *L. mexicana* and *L. braziliensis* [15, 56]. Finally, based on *in vitro* assays of the proliferative capacity of sensitized lymphocytes, cross-reactivity could easily be demonstrated in the mouse between *L. enriettii* and *L. tropica* (Al-Zubaidi S.M. and Mauel J., in preparation), and in man between *L. donovani* and *L. tropica* [71]. These few examples indicate that, within one given host species, cross-reactivity can be observed between both pathogenic and non-pathogenic parasites.

In spite of this immunological cross-reactivity, however, it is usually accepted that immunity against one leishmanial species confers no protection against another one (there are a few minor exceptions to this rule). For instance, immunity against *L. donovani* does not protect against *L. tropica*, nor does infection by *L. tropica* vaccinate against *L. donovani* [36, 47]. Individuals recovered from infection by *L. t. minor* are not immune to *L. t. major* [36]. Cross-immunity is lacking between

at least five subspecies of *Leishmania* infecting man in the New World [42]. Contrary to the above observations, a well-documented case of cross-protection appears to be that reported to occur between *L. mexicana* and *L. tropica* in both man and animals [1, 2, 57].

Experiments have been performed in a model of immunoprophylaxis in the mouse, in which the effect of prior inoculation of *L. enriettii* (non-pathogenic for this host species) on the subsequent infection by *L. tropica* has been investigated (Al-Zubaidi S.M. and Mauel J., in preparation). To this effect, animals from susceptible genetic background (with respect to *L. tropica* infection) were injected with the vaccinating agent (*L. enriettii*) incorporated in Freund's complete adjuvant. Sensitized lymphocytes explanted 7 to 30 days later displayed a high level of reactivity to parasite antigen as measured in an *in vitro* proliferation assay, when cultured in the presence of either *L. enriettii* or *L. tropica*, indicating a considerable level of immunological cross-reactivity between both parasite species. When such vaccinated animals were challenged with *L. tropica*, only partial protection was obtained, however, as evidenced by a reduced lesion size and an increase in the number of recovering animals after two months.

As far as the human situation is concerned, the absence of cross-protection in spite of the immunological cross-reactivity demonstrable between leishmanial species appears to be the rule, and constitutes a most puzzling phenomenon. It is usually asserted that the classical tests of humoral and cellular immunity detect only "irrelevant" antigens, that is antigens eliciting an immune response that is not protective. If this view is correct, the nature of the "relevant" antigens still remains elusive. Considering the intracellular nature of the parasite, it would be conceivable that "protective" antigens might be released by the microorganisms only after they have taken residency inside their host cells. Under these conditions, immune reactions detected by skin testing or other assays using killed parasites or their extracts might indeed be irrelevant.

V. Conclusions: some points to be considered in the perspective of vaccine development against Leishmanial infections

Some leishmanial infections, particularly the cutaneous disease known as the *Oriental sore*, are self-limited and relatively henign. Although an effective vaccine other than the virulent parasite itself would undoubtedly

be of great value in this case, it is far more important to conceive and produce vaccines against the more serious forms of *leishmaniasis* such as *Kala-azar* and *Espundia.* The following considerations are presented in this perspective.

1. As reported above (Section II.C.1), immune resistance against established leishmanial infections appears to depend on cell-mediated mechanisms rather than on humoral factors. Unfortunately, no information is available as to the nature of the mechanisms by which *recovered* individuals are protected against reinfection. Three observations suggest, however, that such mechanisms also involve the agency of cells: *a*) fresh normal human serum is lytic towards all species of *Leishmania* promastigotes *in vitro*, yet man is susceptible to infection; *b*) antibody titers in people recovered from Oriental sore are very low, yet such individuals are immune against reinfection; *c*) the onset of immunity against Kala-azar correlates with the appearance of a positive delayed skin test. It would thus seem that a good anti-leishmanial vaccine would be one that stimulates cellular rather than humoral immunity.

2. Effector mechanisms against leishmanial infections appear to involve macrophage activation. If the observation is correct that activated macrophages from certain animal species or strains are intrinsically deficient in their capacity to destroy the intracellular microorganism (Section II.C.3), and if such a situation also occurs in certain genetically predisposed humans, it is to be expected that such individuals will not benefit from vaccination. In Kala-azar, it appears that failure to heal spontaneously is *not* due to a macrophage defect, since recovered people are protected against reinfection. Rather, parasite survival appears to depend on its capacity to prevent the development of an appropriate cell-mediated response.

3. As pointed out in Section IV, observations in humans and experiments with animals suggest that no or little protection is afforded by natural or artificial infection with heterologous leishmanial species (except in rare instances), by subclinical infections induced by inoculation of subthreshold numbers of virulent homologous parasites, by homologous attenuated parasites, or by their extracts. The general conclusion from these experiments is that protection can be obtained only by inoculation of homologous parasites that are permitted to produce an overt infection, and by letting this infection run its full course. If these observations

are correct, it is to be expected that best protection against the lethal forms of leishmaniasis will be achieved by the use of homologous parasites treated in such a way that they will still induce an infection, which should naturally be of a self-limited nature. Such "modified" parasites might be obtained from wild reservoirs, as suggested by experiments described in Section IV.B. Alternatively, they might be selected by cloning *in vitro* (provided that adequate means of testing the populations thus obtained be available), or induced by mutagenic procedures.

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PROSPECTS FOR VACCINATION AGAINST AFRICAN SLEEPING SICKNESS

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INTRODUCTION: SLEEPING SICKNESS

Sleeping sickness is caused by genetic variants of the parasitic flagellate *Trypanosoma brucei*. This species naturally infects a range of wild and domestic mammals in Africa and is transmitted by tsetse flies (*Glossina* spp.). The parasite variants, distinguished by their ability to infect man, are given subspecific designations. *T. b. gambiense* causes the more chronic West African disease and is transmitted by riverine flies such as *Glossina palpalis* in moist savanna and forest regions. *T. b. rhodesiense* causes the more acute East African form of the disease; its vectors are drier savanna flies such as *Glossina morsitans*. *T. b. brucei*, which does not infect man, is widespread throughout the tsetse belt of Africa, but the sleeping sickness trypanosomes occur in about 200 small endemic foci of great antiquity, scattered over 38 African countries south of the Sahara.

Approximately 10,000 new infections of sleeping sickness are reported annually but some 50 million people are at risk from epidemic resurgence of the disease. An increasing number of outbreaks have occurred in recent years, notably in Sudan, Cameroon, Angola and Uganda. Epidemics of sleeping sickness arise when increased transmission of the trypanosomes occurs in an endemic area. Resurgence may be due to an increase in contact between man and fly as a result of changes in climate or vegetation, or due to changes in human habits or habitat, often as a result of population movements. Another possibility is that the trypanosomes themselves have undergone genetic change.

Human sleeping sickness is almost invariably acquired as the result of a bite from a tsetse fly whose saliva contains the infectious metacyclic stage in the life cycle of the trypanosome. The trypanosomes are deposited in a pool of blood which has leaked from capillary vessels ruptured by the cutting action of the vector's proboscis. At this site in the dermis the parasites multiply and provoke a strong inflammatory response from the host. A swelling or "chancre" forms as invading leucocytes (neutrophils first, then lymphocytes and, lastly, macrophages) crowd into the area and extravasated fluid accumulates; this reaction does not occur with an uninfected tsetse bite. From the chancre the trypanosomes find their way into the lymphatics and then into the bloodstream. From the blood the parasites escape into the soft connective tissues, continuing to multiply by binary fission as long slender flagellates. The connective tissue of the choroid plexus may be invaded and from there the nervous system via the cerebrospinal fluid.

The blood of sleeping sickness patients and infected laboratory animals shows a relapsing parasitaemia, surges of dividing slender trypanosomes giving way in remission to diminishing numbers of parasites which are short and stumpy in form; it is these short forms that are infectious to the tsetse fly should the vector feed while these are present in the blood. After a fall in trypanosome numbers — which may appear to clear the blood entirely — a recrudescence of parasitic numbers follows and the process is repeated over and over again until the patient or animal dies, or is successfully treated by administration of a suitable drug.

With each renewed appearance of trypanosomes in the blood, the patient experiences fever, headache and joint pains. As the infection progresses typical lesions develop in the brain, heart and small blood vessels. Clinical signs include insomnia and over-excitability. Later these symptoms are replaced by manifestations of central nervous system lesions, eventually resulting in coma and death. The clinical picture presented by *Trypanosoma brucei rhodesiense* infection is that of a more acute disease than that caused by *T. b. gambiense* with high parasitaemia and severe symptoms from the onset. But although the East and West African diseases are quite distinct from the clinical and epidemiological point of view, their essential pathology and treatment principles are the same, and both are fatal if left untreated.

There is no obvious radical solution to the prohlem of controlling sleeping sickness. Control of the tsetse vector is restricted by the high incidence of reinfestation unless control projects are followed by agricul-

tural land development so that the habitat of the flies has been permanently removed, and this happens only rarely. The present policy for prevention and control is based on maintaining endemicity at a low level by regular surveillance and drug treatment of the population at risk. Where possible this surveillance is combined with anti-vector measures to reduce contact between man and fly.

But surveillance is costly to maintain, and disillusion with insecticide spraying, together with the problems raised by drug prophylaxis (resistance, toxicity and production costs) have frequently raised the question of whether vaccination might not be a more effective way of controlling the disease. The possibility of immunizing people against trypanosomiasis has, however, long been regarded as a forlorn hope. The root of the problem is the trypanosome's remarkable ability to evade its host's immune response by undergoing antigenic variation.

The mechanism of trypanosome antigenic variation

Since the early years of this century the fluctuating parasitaemia of chronic sleeping sickness trypanosome infections has been ascribed to the parasite's ability to undergo a change in its antigenic nature. In this way the trypanosome survives the host's antibody response and can multiply unhindered until antibodies are produced against the new phenotype and the parasite population again dwindles.

A new variable antigen type multiplies to give rise to each parasitaemic peak. These peaks occur at intervals of 1 to 8 days in sleeping sickness patients. Most of our understanding of what goes on in human sleeping sickness infections, however, is based on observations on experimental infections in laboratory animals.

Within the last few years the use of refined serological techniques for the identification of different variable antigen types (VATs) has changed our picture of what is happening in antigenic variation quite considerably. Antigenic change used to be regarded as an induced event, a substantial number of parasites in the trypanosome population adapting to the presence of host antibody by changing its VAT and thus being able to continue the infection (summarised by Vickerman, 1974). A programmed sequence of VAT changes was envisaged, starting from a "basic antigen" — the VAT of the first trypanosome population to grow up in the mammal and probably the same as that of the metacyclic popu-

lation inoculated by the tsetse fly. This picture of antigenic variation was largely based on the study of qualitative changes taking place in antibody production as assessed by agglutination reactions using trypanosome populations derived from infected mouse blood and assumed to be uniform in their VAT (Gray, 1965, 1975).

By working with clone lines of trypanosomes and studying changes in these genetically defined populations in laboratory animals, it has been possible to examine more critically the roles of induction and selection in antigenic variation and to sort out the contributions of phenotypic and genotypic change on the part of the trypanosome to this process. Introduction of the indirect fluorescent antibody test and modification of the complement-dependent trypanolysis reaction have, moreover, enabled identification of the VAT of individual trypanosomes and the monitoring of changes in the VAT composition of trypanosome populations (Van Meirvenne *et al.*, 1975a). As a result of these advances a different story has emerged.

For a given clone of trypanosomes there appears to be a limited repertoire of VATs that can be displayed. Trypanosome clones having similar repertoires are said to belong to the same serodeme (Van Meirvenne *et al.*, 1975b). The relative constancy of repertoires during passage in the laboratory, plus the similar order of appearance of their VATs in the blood of infected animals, indicates that selection of random point mutations could not be the source of diversity in trypanosome antigenic variation. The obvious conclusion is that the serodeme reflects the genotype of the clone and that the different VATs are the phenotypic manifestations of different genes.

Yet the way in which new VATs arise is very suggestive of a mutational process. If we examine a trypanosome population by immunofluorescence using monospecific antisera to a range of homologous serum VATs, we find that, although the major part of the population may display the same VAT, in addition to these "homotype" trypanosomes there is a minority population of "heterotypes" — trypanosomes that belong to different VATs. After elimination of the homotype by the host's immune response, one of these heterotypes will emerge as the homotype of the next parasitaemic peak, the others will give rise to future homotypes in their turn. Of course the picture is not always as simple as this; when one homotype is in the descendancy, another may be ascending so fast that the parasitaemic peaks tend to coalesce, and this situation appears to be more common in fly transmitted infections of

natural hosts. In syringe passaged clone-derived infections of *Trypanosoma brucei,* heterotypes can be detected at the rate of 1 in 10,000 trypanosomes (Van Meirvenne *et al.*, 1975a). The apparently spontaneous way in which heterotypes arise in non-immune hosts or even *in vitro* is suggestive of a process of genetic mutation hut this was discounted above on grounds of VAT repertoire constancy; it may be further discounted on grounds of variable antigen structure as discussed below. There is, in fact, now a good deal of evidence to suggest that transposition of genetic elements in the trypanosome nucleus can account for the generation of heterotypes (Borst *et al.*, 1981). The question why and how such genes or their promoters jump is one of much wider import in biology today but one for which, as yet, we have no answers.

The discovery of heterotypes was a major blow to the idea of an antibody-induced series of programmed changes of VAT being at the root of antigenic variation. The processional order of VATs suggested by studies on agglutinin production (Gray, 1965, 1975) we now know applies only to homotypes; heterotypes arise in all populations independent of this sequence and what enables one particular heterotype rather than another to be next in succession we do not know. It appears that some VATs arise as heterotypes more frequently than others (Van Meirvenne *et al.*, 1975a; Miller & Turner, 1981), also that some VATs are able to overgrow others with which they are in competition, possibly by virtue of shorter trypanosome doubling time (Seed, 1978). In addition, having been generated, some VATs are less stable than others and this appears to be related to their tendency to produce non-dividing stumpy forms (Le Ray *et al.*, 1977; Barry *et al.*, 1979a). All these factors may add together to explain why certain VATs, the so-called "predominant" antigenic types always appear early in an infection, whether it was initiated by fly bite or syringe. What seems to be certain now is that host antibody plays a purely selective role in antigenic variation and not an inductive one.

Antigenic variation has long provided the main reason for despondency about prospects of vaccination against both human and animal trypanosomiasis. Although each VAT in the form of its purified variable antigen is capable of eliciting a strong protective immuntiy in an experimental animal (Baltz *et al.*, 1977), immunity is VAT-specific and heterologous VATs are able to multiply in the protected animal. We are, as yet, unable to state the total number of VATs in any *Trypanosoma brucei* serodeme but in the closely related *T. equiperdum* of horses this number

may exceed 100 (Capbern *et al.*, 1977). With so much potential for variation in a single trypanosome, the problem of protecting a host against the VATs of a battery of local serodemes appears prohibitive. We shall consider, however, some of the suggestions that have been put forward with a view to surmounting this enormous obstacle. For earlier recent reviews on this subject, largely from the veterinary standpoint, the reader is referred to Murray & Urquhart (1977), Holmes (1980) and Murray *et al.* (1980).

Vaccination against common antigens or common determinants on the variable antigen molecule.

The variable antigen of the trypanosome is present as a coat on its surface membrane and this coat envelops the entire body and flagellum (Vickerman, 1969). The coat is a monomolecular layer of glycoprotein, the structure of which varies from one VAT to another (Cross, 1975). When the trypanosome enters the vector, or is grown *in vitro* at 25° in the form corresponding to the early vector stage, both coat and VAT specificity are lost (Barry & Vickerman, 1979) and procyclic forms derived from various *T. brucei* serodemes display common surface antigens (Seed, 1964). After multiplication and migration in the vector, the trypanosomes end up in the fly's salivary glands and the trypanosomes reacquire the coat as they differentiate into the infective metacyclic stage to be discharged in the vector's saliva (Vickerman, 1974).

Each species of surface coat glycoprotein (VSG, variable antigen) consists of a single polypeptide chain with its carboxy (C)-terminus attaching the molecule to the surface membrane proper and its amino (N)-terminus directed outwards. The two parts of the molecule can conveniently be separated by trypsin cleavage and the carbohydrate groups are found to be attached to the C-terminus (Cross & Johnson, 1976; Johnson & Cross, 1979). The specificity of the antigen resides in the amino acid sequence of the N-terminus and the tertiary structure of the protein that this creates. The differences in N-terminus sequence between different VSGs of trypanosomes belonging to the same serodeme are so substantial that they constitute another good argument against mutation as the generator of VAT diversity, as there are no common sequences (Bridgen *et al.*, 1976). Cross-reacting VATs belonging to different serodemes are

known, however, and these so-called iso-VATs obviously have antigenic determinants, and therefore some amino acid sequences, in common (Van Meirvenne *et al.*, 1977).

Unlike the N-termini of VSGs belonging to the same serodeme, the C-termini show some serological cross reactivity which appears to be related to common glycopeptides in this portion of the molecule (Barbet & McGuire, 1978; Holder & Cross, 1981). Such common antigenic determinants are inaccessible to antibodies in the living trypanosome and probably play a part in attaching the VSG to the membrane beneath.

In addition to the variable antigen, the bloodstream trypanosome contains other antigens which are common to populations of trypanosomes of different VAT, to trypanosomes of different serodemes, and in some cases to other stages in the *Trypanosoma brucei* life cycle, such as the procyclic stage which can readily be grown in bulk *in vitro* (Le Ray, 1975). These "common" or "stable" antigens belong to internal organelles or may be present on the surface but covered by the variable antigen monolayer (Barry & Vickerman, 1979). They are released into the blood or tissue fluid each time a homotype is eliminated by host antibody, and elicit an IgG response which is useful in serodiagnosis (as detection of antibodies to these common antigens in a patient's serum is indicative of trypanosome infection) but is of no avail in protecting the host against infection. Thus it is possible to hyperimmunize an animal against these common antigens (e.g. using procyclic trypanosomes and Freund's complete adjuvant) and it can still be infected with *T. brucei*. The reason why is obvious: the common antigens being concealed beneath the surface coat are inaccessible to host antibodies. For similar reasons, plans to hyperimmunize animals with the C-termini of variable surface glycoproteins, as these have common determinants in different VATs, will doubtless be ineffectual.

An interesting side issue of these investigations, however, is the possibility that hyperimmunization of the host against the common antigens of procyclic forms may prevent transmission of trypanosomiasis by the fly (Murray *et al.*, 1980b). Such antigens become exposed as the bloodstream trypanosomes transform to the procyclic stage (Barry & Vickerman, 1979) and ingested antibody can retain its specificity for up to 4 days in the vector's gut (Cunningham *et al.*, 1962). Such hyperimmunization would be comparable to use of the anti-gamete vaccine in malaria control.

Non-specific immunization by treatment with immunostimulants

There is accumulating evidence that certain microorganisms, notably *Mycobacterium bovis*, *Bordetella pertussis* and *Corynebacterium parvum* confer on mice some protection to animal parasites, especially intracellular protozoa and a range of helminths (reviewed Cox, 1981). This protection may be due to enhancement of specific immunization, the restoration of immunocompetence to animals rendered immunodeficient by the parasite itself, or the enhancement of non-specific immunity. In infections with the African trypanosomes the enhancement of specific immunization would not prevent the trypanosome undergoing antigenic variation, but may be of limited benefit. Although there is a considerable amount of evidence for immunodepression in sleeping sickness (Greenwood *et al.*, 1973) and trypanosome infections of laboratory animals (Hudson & Terry, 1979) the possibility that this may extend to the parasite itself (Sacks & Askonas, 1980), has not been fully investigated, so we do not know if restoration of immunocompetence might be advantageous to the host in combatting trypanosome infections, though it may help to counter intercurrent infections which are frequently a cause of death in sleeping sickness.

Experiments by Murray & Morrison (1979) demonstrated increased survival time of both susceptible (A/J) and more resistant (C57B1/6J) strains of mice when BCG, *Bordetella pertussis* or *Corynebacterium parvum* were administered before or at the time of infection with *Trypanosoma congolense*. Increased survival time appeared to be the result of delayed onset of parasitaemia and a subsequent reduced level of parasitaemia, and complete protection was never obtained.

There would seem to be little point in pursuing this approach in human sleeping sickness infections, however, as not only is protection far from perfect but the pathological effects of the immunostimulants are such that they could not be tolerated in any vaccination scheme.

Any vaccination plan which seeks to enhance non-specific immune mechanisms to sleeping sickness trypanosomes is based on a false premise, as trypanosomes appear to avoid such mechanisms, again by virtue of their surface coat. Thus uncoated procyclic trypanosomes are lysed rapidly in fresh non-immune sera of susceptible hosts while the coated bloodstream stages themselves are unaffected. Lysis of uncoated forms probably results

from activation of complement by the alternate pathway as it occurs in the absence of specific antibody and the activity is heat (56°) labile (Rifkin, 1978). The coated stages of *T. brucei*, and purified variable antigen itself, are said to be able to decomplement serum via the classical pathway, depleting C_1, C_2, C_4 and to a small extent C_3 (Musoke & Barbet, 1977). Despite this activation, the trypanosomes are not lysed. It is possible that the pathway, though activated, is not completed, and a membrane attack complex is not formed or, if it is, its exposed hydrophobic peptides are unable to insert into the surface membrane lipid bilayer as the hydrophilic N-termini of coat glycoprotein molecules bar their way. VAT-specific antibody is necessary for complement-mediated lysis of the coated trypanosome and such lysis probably provides a major destructive mechanism for clearing trypanosomes from the blood of an infected host.

In addition to preventing complement-mediated lysis, the surface coat also appears to discourage phagocytosis in the non-immune host. Uncoated trypanosomatids are readily phagocytosed by peritoneal macrophages *in vitro* whereas the uptake of bloodstream *T. brucei* under similar conditions is negligible. In the presence of homologous (VAT-specific) antibody, however, uptake is avid (Takayanagi *et al.*, 1974a, b). *In vivo* ^{75}Se-labelled *T. brucei* are rapidly cleared from the circulation of mice immunized by previous infection and treatment, the Kupffer cells of the liver removing most of the parasites within minutes of injection (McAskill *et al.*, 1980). In non-immunized mice, however, the labelled trypanosomes remain in the circulation. Opsonization is probably another major effector mechanism in specific immunity to African trypanosomiasis, and the mononuclear phagocyte system becomes greatly expanded in infected hosts (Murray *et al.*, 1974). The relative importance of lysis and phagocytosis as effector mechanisms is debatable and may vary from one host species to another. What appears to be certain is that in the susceptible host neither process occurs in the absence of VAT-specific antibody, and neitber process plays a part in non-specific immunity.

VACCINATION BY INTERRUPTION OF THE SEQUENCE OF VARIABLE ANTIGEN TYPES.

As the variable antigens are the only trypanosome components known to confer protective immunity on a host, and insofar as we know any

one VAT is capable of causing some disease, specific immunity against all VATs of all local serodemes is clearly what is required of a vaccine against sleeping sickness. An alternative approach would be to inhibit the process of antigenic variation itself. The first probability could clearly be a daunting task to pursue, if not only the number of serodemes but also the number of VATs in a serodeme is large. Hopes of achieving the second possibility were raised, however, by the finding of an order of appearance of VATs in infected animals, that certain "predominant" VATs were always expressed early in the course of an infection, and especially that following tsetse transmission the trypanosomes reverted to a "basic antigen" which started the sequence in a new host (Gray, 1965, 1975). If the sequence could be halted at an early stage, by rendering the host solidly immune to one or more of the predominant antigens, or better still to the basic antigen, then antigenic variation might cease to be a problem.

We have seen, however, that although the procession of VAT homotypes in the early stages of an infection may give the impression of a programmed sequence, the generation of heterotypes does not occur in a sequential fashion; passive immunization of experimental animals against certain predominant VATs results simply in those VAT homotypes being omitted from the sequence, other VAT heterotypes growing up to take their place. Recent work on the relapse variants arising from a particular VAT clone has indicated that heterotype VATs arise in a statistically definable order of priority, and that this order is different for each parent VAT (Miller & Turner, 1981), but the fixed pattern of heterotype production which would be required for vaccination based on VAT sequence interruption does not occur.

Of course, the most satisfactory point at which to halt infection would be immediately after deposition of the metacyclic trypanosomes in the skin of the host by the tsetse fly. If all variants revert to a basic VAT in the tsetse fly, then protection of a potential host against the basic VATs of local serodemes should prevent the generation of heterotypes and hence survival of the trypanosome. Unfortunately, recent work in our laboratory has shown that even when the infecting fly ingests a clone population of trypanosomes, not only are trypanosomes of the first patent parasitaemia heterogeneous with respect to VAT, but the metacyclic population injected by the fly is also heterogeneous (Le Ray *et al.*, 1978; Barry *et al.*, 1979b; Barry & Hajduk, 1979; Hajduk *et al.*, 1981; Hajduk & Vickerman, 1981a, b). There is no basic antigen to which all trypa-

nosomes revert during cyclical transmission. Jenni (1977, 1979) has reported serodeme-specific basic antigens in cyclically transmitted *T. brucei* infections, but there can be little doubt that the uniform staining of metacyclics in the immunofluorescence test that he obtained was due to the use of polyvalent antisera. Although in our earlier experiments metacyclic heterogeneity was demonstrated by using similar antisera, but appropriately diluted to give VAT-specific staining or lysis, our more recent experiments using VAT-specific monoclonal antibodies have confirmed VAT heterogeneity (Crowe *et al.*, 1981). The last hope for vaccination based on VAT sequence interruption must therefore be abandoned.

VACCINATION AGAINST METACYCLIC VARIABLE ANTIGEN TYPES OF LOCAL SERODEMES.

The finding that the metacyclics extruded by a tsetse fly, which had ingested trypanosomes belonging to a single serodeme, were heterogeneous with respect to VAT, raises two important questions. First, is the number of metacyclic VATs (M-VATs) present constant for a given serodeme and, second, how many serodemes of sleeping sickness trypanosomes are to be found in nature? If the M-VAT repertoire is composed of just a few VATs which are constant for a given serodeme and if the number of serodemes circulating in the different endemic foci is also small then vaccination based upon protection against a few variable antigen types may yet prove feasible.

At present we can only offer tentative answers to these questions. We have identified 3 M-VATs in each of two serodemes (AnTAR 1 and ETAR 1) of *T. brucei* but these account for only 30% (AnTAR 1) and 65% (ETAR 1) of the total M-VATs, so we presume that at least 4 M-VATs are present in the metacyclic population of either serodeme; the total number could be greater. The same M-VATs are expressed at roughly the same percentages, however, in each case, regardless of the age of the infection in the fly, or of the VAT originally ingested by the vector, *Glossina morsitans morsitans.* Strangely enough, the VAT ingested by the fly (I-VAT) does affect the composition of the first patent parasitaemia in experimental mice. In this parasitaemia, the first VATs to be detected are the M-VATs, followed by the I-VAT, followed by a characteristic spectrum of predominant VATs which are presumably characteristic relapse VATs of the I-VAT (Hajduk & Vickerman, 1981b).

What is responsible for the constancy of M-VAT composition of the metacyclic population we do not know. With L. Tetley and J.S. Crowe we have recently been able to demonstrate that the metacyclic trypanosomes are heterogeneous with respect to VAT when they arise from uncoated trypomastigotes attached to the microvilli lining the fly's salivary gland. Peroxidase conjugated monoclonal M-VAT-specific antibody binds to some coating attached trypanosomes, but not to others. It seems unlikely that antigenic variation occurs in the differentiated metacyclic trypanosomes, as these do not divide, but the assumption that antigenic variation is the prerogative of dividing trypanosomes (i.e. slender bloodstream forms) is not at present wholly justifiable.

Preliminary examination of M-VATs of stocks of the ETAR 1 serodeme isolated at different times over a period of two decades from the endemic Rhodesian sleeping sickness focus to the north of Lake Victoria suggests that the M-VAT repertoire may drift with time in the field, new M-VATs appearing while others disappear from the metacyclic population but remain in the total serodeme repertoire as bloodstream VATs. It appears, however, that within a single epidemic the M-VAT repertoire does not change, and this could be of practical importance. Alteration of the M-VAT repertoire in the field may be the result of rapid VSG gene evolution and genetic recombination between different serodemes. At present genetic recombination as a result of a sexual process in trypanosomes remains an interesting possibility deserving further investigation (Tait, 1980).

The number of serodemes of *T. brucei* circulating in nature appears to be large (Gray, 1972; Van Meirvenne *et al.*, 1977) but for the sleeping sickness subspecies, what incomplete information we have suggests that the number of serodemes may be quite few. Thus Gray (1972), Jones *et al.* (1981) and Le Ray (unpublished) found a considerable number of VATs in common between isolates of *T. b. gambiense* from several West African countries. Le Ray's detailed study of predominant VATs showed one serodeme to be very widespread over *T. b. gambiense* foci; he also detected one minor serodeme. A restricted serodeme number seems likely for *T. b. rhodesiense* in its South Ugandan focus; here recurrent epidemics appear to have been due to resurgence of the same serodeme (Barry, unpublished) although, again, there is probably at least one other minor serodeme present in the area. This work on serodeme recognition has largely turned on the detection of defined VATs in the sera of rabbits chronically infected with a particular isolate. This approach is fraught

with problems. Not all isolates of sleeping sickness trypanosomes — *T. b. gambiense* in particular — are readily adapted to laboratory animals. It is possible that in such artificial hosts not all the VATs in the trypanosome's repertoire are expressed; we have a documented example of the VATs expressed being determined by the experimental host in the rodent-infecting stock of *T. vivax* (De Gee *et al.*, 1981; Barry (unpublished).

If vaccination with M-VATs against local serodemes is to be a practical possibility some means of producing the metacyclic antigens in bulk has to be available and a more rapid procedure for identifying the relevant serodemes than the present laborious serological technique, described above, is desirable.

Recent years have seen the long awaited advancement of cultivation *in vitro* of bloodstream forms of *T. brucei* including its sleeping sickness variants (Hirumi *et al.*, 1977, 1980; Hill *et al.*, 1978; Brun *et al.*, 1979) and the possibility of cloning *in vitro* (Hirumi *et al.*, 1980). Metacyclic VATs are extraordinarily labile after cloning (Le Ray *et al.*, 1977) but their bloodstream equivalents obtained from syringe passaged stocks are more stable (Barry *et al.*, 1979b) and could be used to initiate cultures. As antigenic variation can occur *in vitro* (Doyle *et al.*, 1980), however, such cultures might still not represent a wholly reliable source of trypanosomes of a given VAT. A better source of M-VAT antigen could he obtained through recombinant DNA tecbnology. Messenger RNA from a trypanosome population of uniform VAT can be used to manufacture complementary DNA (c-DNA) using the enzyme reverse transcriptase and this c-DNA cloned in *Escherichia coli* or *Bacillus subtilis* using a plasmid vector (Hoeijmakers *et al.*, 1980). The bacterium will then produce the specific VSG and can be grown in bulk to provide a source of antigen for vaccination.

Recombinant DNA technology could also help to provide a more rapid method for serodeme identification. It has been found that the extracted nuclear DNAs (n-DNA) of different trypanosome stocks when subjected to digestion with restriction enzymes and electrophoresed in agarose gels give characteristic band patterns of genomic fragments which can be used to compare variable antigen gene repertoires. ^{32}P-labelled c-DNA probes prepared against individual VATs, as described above, will hybridise with homologous fragments of the genome and this hybridization can be revealed by autoradiography. The restriction digests are

"blotted" onto nitrocellulose filters for hybridization and the resulting autoradiogram is termed a "Southern blot". Identical serodemes give identical Southern blot patterns with the different c-DNA probes, so this technique could be used to identify serodemes given enough trypanosomes in an isolate to obtain a nuclear DNA preparation.

Southern blot analysis has already yielded valuable information on variable antigen gene expression and evolution. Each c-DNA probe hybridises to a "family" of related genes (Hoeijmakers *et al.*, 1980); homology between members of a family increases from the 5' end of the messenger RNA to the 3' end (Borst *et al.*, 1980; Frasch *et al.*, 1980; Borst *et al.*, 1981). This suggests that VSG genes evolve by a conventional duplication-divergence mechanism, but that the 5' half accumulates mutations more rapidly than the 3' half. This is what one might expect because it is the 5' half that codes for the antigenic determinants seen by host antibodies, i.e. the N-terminus of the VSG molecule, whereas the 3' half encodes the C-terminus which attaches the molecule to the membrane beneath. Use of c-DNA probes also shows that expression of several VSG genes is accompanied by duplication of the gene and its transposition to another DNA segment. This transposed "expression linked copy" is not found in n-DNA of all VATs of the serodeme, only in the VAT homologous n-DNA. Some VSG genes do not appear to be duplicated when expressed, and these are expressed early in the infection, i.e. as predominant VATs. These genes undergo rearrangements unrelated to expression and represent a particularly unstable subset. It has been postulated that this subset is capable of undergoing rapid evolution (Borst *et al.*, 1981).

For our vaccine plans we need to know much more about VAT repertoire evolution, especially M-VAT evolution and how rapidly it occurs in the field. This knowledge can only come from a study of carefully documented sequential isolates made over a known time period. We also need to know how the M-VAT genes fit into the picture that we have to date of the trypanosome genome as outlined above. We do not know, for example, whether the M-VAT repertoire genes are under separate control from the bloodstream VAT repertoire. The extraordinary reappearance of the I-VAT after the M-VATs in the first patent parasitaemia suggests independent control of the M-VATs. At present rates of progress we can expect most of these questions to be answered in the next few years.

Conclusions

Antigenic variation may not prove an insurmountable barrier to vaccination against human sleeping sickness if plans for vaccination based on the use of metacyclic variable antigen types are explored and if (1) the number of variable antigen types in the metacyclic trypanosomes of a serodeme can be determined and proves to be relatively few; (2) the number of serodemes circulating in a given focus is small (preliminary results suggest that it may be); (3) the rate of evolution of metacyclic variable antigen genes in the field is not rapid, so that new metacyclic variable antigen types are unlikely to arise during the course of an epidemic. The technology is now available not only to enable us to fill these gaps in our knowledge but also to produce metacyclic antigen in bulk and also identify serodemes rapidly. Research over the next five years should decide whether or not these plans are feasible.

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THE DEMONSTRATION OF IMMUNITY TO *SCHISTOSOMA MANSONI* IN THE MOUSE AND ITS CORRELATION WITH *IN VITRO* FINDINGS

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INTRODUCTION

The encouraging progress in understanding the basis of resistance to reinfection in schistosomiasis during the last decade, may be summarised in the following key findings.

Firstly, from *in vitro* studies, a variety of immune mechanisms have been described which kill or damage young schistosomula in culture. All of these systems are dependent on specific anti-schistosome antibody acting in cooperation with complement and/or various effector cells (Table 1).

A second important finding which is also a consequence of the *in vitro* approach relates to the ability of the schistosome to survive in the face of the host's immune response. Some of the *in vitro* effector systems shown in table 1, notably antibody and complement, and antibody and eosinophils, have been used to demonstrate that the young schistosomula rapidly acquire the ability to evade immunity (Clegg and Smithers, 1972; Dessein *et al.*, 1981; McLaren and Incani, 1981). Although the mechanisms on which evasion is based are still in contention (Moser *et al.*, 1980; McLaren and Terry, 1981), there is no doubt that within a few hours of transformation from cercariae to schistosomula, schistosomes become less susceptible to immunity, and by the time they are 4 days old or have reached the lung stage, they are no longer recognised by antibody and are therefore no longer susceptible to antibody-dependent mechanisms (McLaren *et al.*, 1975; Goldring *et al.*, 1977).

TABLE 1 — *Effector mechanisms which kill or damage young schistosomula* in vitro.

1. IgG+complement	:	Clegg and Smithers, 1972
2. IgG+eosinophils	:	Butterworth *et al.*, 1977
3. IgG+eosinophils+complement	:	Anwar *et al.*, 1979; McLaren and Ramalho-Pinto, 1979
4. IgG+neutrophils+complement	:	Anwar *et al.*, 1979; Incani and McLaren, 1981
5. IgE+eosinophils	:	Capron *et al.*, 1981
6. IgE/complexes+macrophages	:	Capron *et al.*, 1977

Progress from *in vivo* studies during recent years has been less successful. There has been a sensible and predictable transition from working with monkeys to syngeneic rodent models, and the passive transfer of resistance with serum has been demonstrated in rats and, although less convincingly, in mice (Phillips *et al.*, 1975; Sher *et al.*, 1975). However, I would rank the most significant finding from animal experiments as the ability of cercariae irradiated at high levels (20-50 Krads) to induce substantial levels of protective immunity (Hsü *et al.*, 1969; Minard *et al.*, 1978; Bickle *et al.*, 1979). Very few cercariae irradiated at this level migrate out from the skin, and the immunity induced in this way is therefore dependent on a single and brief stage of parasite development.

During this intensive period of research however, there has been a distinct dichotomy of approach, with little meaningful dialogue between *in vitro* workers and their *in vivo* colleagues. Thus any serious attempt to relate *in vitro* findings with the situation in the intact animal has been largely ignored. I believe it is now time to look more closely at our animal models and ask specifically if we can relate *in vitro* findings with the immunity to reinfection which can he demonstrated *in vivo*. In this paper I shall confine my remarks to the *Schistosoma mansoni*/mouse model.

Immunity in Infected Mice

As I have already mentioned, the young schistosomula is the stage of the parasite which rapidly acquires the ability to evade the immune

response. The 4-5 day old lung worm is no longer recognised by anti-schistosome antibody and consequently it is not susceptible to damage by antibody-dependent mechanisms *in vitro*. Neither is it susceptible to cytotoxic T-cells (Butterworth *et al.*, 1979).

If these findings are a true reflection of the situation in the intact animal, then specific acquired immunity in the host must act during the first few hours of the schistosomulum's existence, i.e. whilst it is in the skin. By the time the parasite has reached the lung stage it is no longer susceptible. Recent findings however, have indicated that in the chronically infected mouse a large proportion of a challenge infection is destroyed after the parasites have passed through the lungs (Dean *et al.*, 1978; Smithers and Gammage, 1980; Blum and Cioli, 1981).

Fig. 1 represents an amalgam of several experiments from my own laboratory. The histograms represent the recovery of a challenge infection from chronically infected mice expressed as a percentage of the recovery of a similar challenge from control mice. The recoveries on day 2 were from the skin site exposed to cercariae, before the schistosomula had begun their migration to the lungs; on day 5 the recoveries were from both the skin and the lungs, before the schistosomula had begun to migrate to the liver; and on day 21, the recoveries were from the liver when all the migrating parasites had arrived in that organ (Smithers and Gammage, 1980).

It is clear that the majority of a challenge infection in the chronically infected mouse is destroyed sometime after day 5, either when the young schistosomes are en route to the liver, or as soon as they arrive in the liver (fig. 1a). Thus in the chronically infected mouse, which has been used as a model for investigating specific acquired immunity, most of the schistosomula of a challenge infection are destroyed at a time when they are no longer susceptible to specific mechanisms *in vitro*.

Dean *et al.* (1981) have recently demonstrated a correlation between the level of resistance developed by different mouse strains and the degree of portal hypertension developed by each strain. That is to say, resistance to reinfection in the chronically infected mouse is apparently related to liver pathology as a result of egg laying and the formation of hepatic granulomas. It has been suggested that either the presence of granulomas stimulates a non-specific inflammatory response which is ahle to destroy schistosomula arriving in the liver, or more likely, the rapid development of a collateral circulation by-passing the liver as a result of

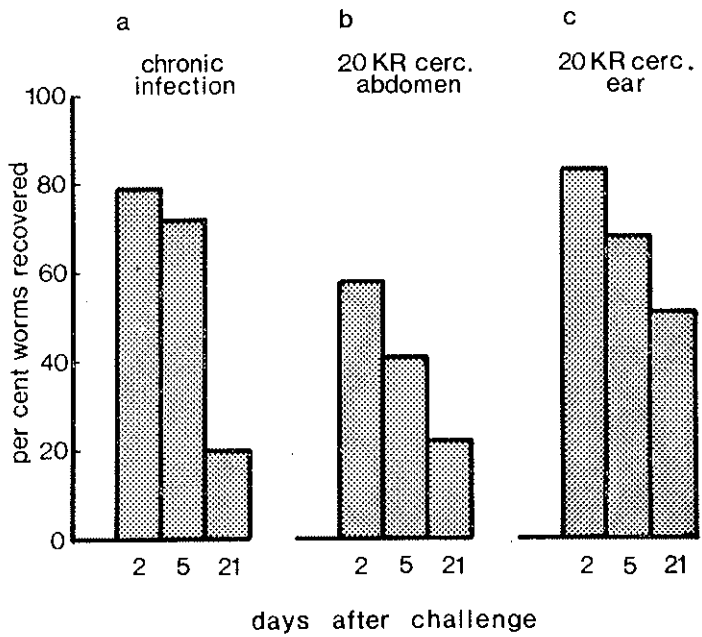

FIG. 1. The recovery of a challenge infection (approx. 130 cercariae) from immune mice expressed as a percentage of the recovery of a similar challenge from control mice.

Recoveries on day 2 were from the skin site of challenge (abdomen) on day 5 from both the skin and lungs; and on day 21 from the liver.

1*a*) An amalgam of 6 experiments using mice infected with 20 cercariae for 15-16 weeks.

1*b*) An amalgam of 16 experiments using mice immunised by exposing the abdomen to 400 20 Krad cercariae and challenging on the same site 4 weeks later.

1*c*) An amalgam of 11 experiments using mice immunised by exposing one or both ear pinnae to 400 20 Krad cercariae and challenging on the abdomen 4 weeks later.

increased portal hypertension, interferes with the arrival of schistosomula in the liver so that they are diverted to other sites in the body (Wilson, 1980).

Supporting evidence that resistance in mice is related to liver pathology comes from reports that mice infected with all male or all female worms (and therefore mice without granuloma formation and liver pathology) fail to develop immunity to reinfection (Dean *et al.*, 1978; Bickle *et al.*, 1979).

In chronically infected mice however, we have consistent evidence for a small but significant level of immunity in the skin, as demonstrated by the low recoveries of a challenge infection on days 2 and 5 (Fig. 1a),

and the possibility is that this pre-lung immunity is a reflection of one of the antibody-dependent mechanisms which have been demonstrated by *in vitro* assays. It could be argued that mice with unisexual infections will also develop a low level of specific immunity which should be demonstrable by reduced recoveries of the challenge on days 2 and 5.

For this reason we infected groups of mice with cercariae derived from one miracidia and 17-18 weeks later each group of mice was challenged with cercariae, and immunity was assayed on days 5 and 21.

Table 2 summarises the results of this experiment; 3 of the 6 groups of mice had developed a clear but partial immunity of about 50%, to the challenge. It was not unexpected that some of the groups failed to show significant immunity, as Smith and Clegg (1979) have clearly demonstrated that different pools of cercariae stimulate widely different levels of immunity. As in this experiment we were dealing with clones of cercariae, each derived from one miracidia, it was no surprise that their immunogenicity varied.

However, whereas by infecting mice with all male or all female worms we were expecting to induce pre-lung immunity, it is clear by comparing the day 5 and day 21 assays, that the immunity which had

TABLE 2 — *Percentage immunity developed after unisexual infection.*

Cercariae			Per cent immunity at 17-18 weeks	
Snail	Sex	No. given	day 5	day 21
A	M	80	13 n.s.	56 $p < 0.01$
B	F	100	10 n.s	18 n.s
E	F	80	14 n.s	17 n.s
F	M	70	19 n.s	56 $p < 0.001$
G	F	135	23 n.s	23 $p < 0.01$
H	F	135	23 n.s	48 $p < 0.001$

Groups of mice were exposed to the cercariae shed from one snail infected with one miracidium and 17-18 weeks later each group was challenged with cercariae. On day 5 after challenge half of each group was assayed for immunity by the skin and lung recovery technique and the remainder assayed for immunity on day 21 by liver perfusion. The immunity is expressed as the percentage reduction of the recovery of a similar challenge from age-matched control mice.

developed was mainly effective after the parasites had passed through the lungs.

This unexpected result demonstrates that post-lung immunity can occur in the absence of liver pathology. It corroborates the finding that a low level of post-lung immunity develops very early after exposure to small numbers of cercariae, at the very beginning of egg deposition and before a collateral circulation has developed (Smithers and Gammage, 1980). Thus, post-lung immunity in infected mice may in part be related to non-specific factors associated with pathology, but an element of post-lung immunity can occur in the absence of liver changes and may be the result of specific acquired factors.

Immunity in Mice Immunised with Highly-Irradiated Cercariae

Mice immunised by exposure to highly irradiated cercariae develop a level of resistance which is comparable to or better than the immunity induced by infection, or that induced by cercariae irradiated at lower levels (Murrell *et al.*, 1978; Bickle *et al.*, 1979; Miller and Smithers, 1980). As most of the cercariae irradiated at the higher level (20-50 Krads) die in the skin a few days after exposure, the immunity induced in this way develops in the absence of liver pathology and almost entirely as a result of a brief exposure to the cercariae/early schistosomula stage.

The pattern of attrition of a challenge infection in mice which have been immunised by exposing the abdominal skin to highly irradiated cercariae and challenging with normal cercariae on the same site 4 weeks later, is illustrated in fig. 1b.

Compared with chronically infected mice, the overall level of immunity is similar but the pattern of attrition differs. A marked level of skin immunity is seen in the immunised mice. Over 40% of the challenge is destroyed by day 2, before the schistosomula have begun their migration from skin to lungs. The increase in killing to 60% which has occurred by day 5, is believed to be the result of a continuing trickle of deaths in the skin. There appears to be a further increment of deaths after the parasites have passed through the lungs.

A phase of parasite attrition in the skin could be a reflection of the immunity that has been demonstrated *in vitro*. Skin immunity in the mouse model may well depend on one or more of the mechanisms effective against the newly-transformed schistosomula in culture. It is an encouraging finding because it suggests that exposure to highly irradiated cercariae induces

a higher level of specific acquired immunity than that induced by a chronic infection (and if we are to produce a vaccine, it will have to improve on the immunity induced under natural conditions).

Examination of the skin site however, 4 weeks after exposure to irradiated cercariae, at the time when the same skin site is normally exposed to the challenge with normal cercariae, demonstrated that the epidermis was still considerably thickened and the dermis contained inflammatory cells, many of which had formed granulomas around the remains of dead irradiated schistosomula from the immunising infection (Miller and Smithers, in preparation).

As these local and temporary changes could account for the high level of skin immunity, we introduced a different mouse model by exposing the ear pinna to irradiated cercariae and then challenging with normal cercariae on the abdomen 4 weeks later. In the ear-immunised mice, the pattern of attrition of the challenge infection is again different (Fig. 1c). The overall level of immunity is reduced compared with the abdomen-immunised mice, and this reduction can be accounted for by a lower level of skin immunity. (A similar pattern of attrition is obtained if the skin sites are reversed and immunisation is via the abdomen and challenge via the ear; a marked level of skin immunity is only evident when immunisation and challenge occur through the same skin site).

The results suggest that the higher levels of immunity following immunisation with highly irradiated cercariae compared with other immunising methods, may in part be due to local changes in the skin site as a result of previous exposure. Such local immunity is unlikely to be long-lasting and is therefore a poor candidate for a vaccine.

The immunity developed by this third mouse model, i.e. immunisation and challenge in a different site, is independent of non-specific factors due to liver pathology and local skin changes, and therefore would appear to be a suitable model for studying specific acquired immunity. Accordingly, it is important to know whether the immunity in this model is predominantly effective in the skin and can be related to mechanisms demonstrated *in vitro*, or whether immunity occurs after the lung stage. The histograms (fig. 1c) suggest that some immunity occurs after the lung. A more accurate method of determining the importance of the skin in the attrition of a challenge infection is to by-pass it by challenging mice intravenously with 5-day old lung worms; the recovery from a lung worm challenge can then be compared with the recovery of a conventional percutaneous exposure to cercariae (Miller *et al.*, 1980).

Table 3 shows that in chronically infected mice, where most of the challenge infection is killed at the post-lung stage, roughly similar levels of immunity are attained against lung worm and cercarial challenges. In contrast, when mice immunised with irradiated cercariae are challenged intravenously or percutaneously, a consistent high level of immunity is seen only against the cercarial challenge. These results imply that in immunised mice, immunity occurs predominantly in the skin, although the slight reduction in recoveries of the lung worm challenge compared to

TABLE 3 — *Cercarial vs lung worm challenge.*

		Percentage immunity after Cercarial challenge	Lung worm challenge
Chronically infected mice	Exp. 1	75 p <0.001	76 p <0.001
	» 2	84 p <0.001	73 p <0.001
	» 3	68 p <0.001	42 p <0.02
	» 4	49 p <0.05	72 p <0.001
20 Krad cerc. immunized	» 5	66 p <0.001	16 n.s
mice (via abdomen)	» 6	79 p <0.001	3 n.s
	» 7	78 p <0.001	7 n.s
	» 8	76 p <0.001	17 n.s
20 Krad cerc. immunized	» 9	n.d	23 p <0.05
mice (via ear)	» 10	43 p <0.02	26 p <0.05
	» 11	54 p <0.001	15 n.s
	» 12	48 p <0.01	20 n.s
	» 13	51 p <0.01	15 n.s

Immune mice were challenged percutaneously on the abdomen with cercariae, or intravenously with 5-day old lung worms recovered from normal mice and derived from the same batch of cercariae used in the percutaneous challenge.

Immunity was assayed on day 21 after cercarial challenge or day 15-17 after lung worm challenge, and expressed as the percentage reduction of the recovery of a similar challenge from age-matched control mice.

Chronically infected mice were infected with 20 cercariae for 15-16 weeks; immunized mice were exposed to 400 20 Krad cercariae on the abdomen or on both ear pinnae and challenged 4 weeks later.

controls indicates that a very small proportion of the total immunity (on the borderline of sensitivity of our assay) is achieved through post-lung killing.

Conclusions

We now have to accept that the mouse model of schistosome immunity is more complex than at first envisaged, with both pre-lung and post-lung mechanisms accounting for resistance to reinfection.

In mice immunised with highly irradiated cercariae, immunity is predominantly pre-lung, effective in the skin. It therefore is most likely to reflect one or more of the antibody-dependent mechanisms which have been shown to kill newly transformed schistosomula *in vitro*. Local changes in the skin however, due to previous recent immunisation, may increase skin immunity. This effect could be due to mechanical factors such as a thickening of the skin, or to the presence of inflammatory cells at the time of challenge, which enhance specific immune mechanisms.

In infected mice, immunity is predominantly post-lung, although a low level of skin immunity develops later during the course of a bisexual chronic infection. The relationship between immunity and liver pathology suggests that in the infected mouse changes in the cell population of the liver, or the development of a collateral circulation, affect the fate of the schistosomula arriving in the liver (it would be surprising if schistosomula were not affected by these changes).

There is preliminary evidence however, that post-lung immunity can also occur in long-standing unisexual infections and also, to a lesser degree in mice immunised with irradiated cercariae. Post-lung immunity in the absence of liver pathology is most puzzling; it appears to be induced specifically, but it acts against a stage of the parasite which can escape specific immune responses. It seems to form an important component of the immune response, at least when adult worms are involved in inducing immunity.

In the future we shall need to dissect the different mechanisms of protection which are evident in animal models and understand each one separately. Only then will we be able to judge the feasibility and potential of inducing resistance by vaccination.

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PERSPECTIVES OF VACCINATION IN CHAGAS DISEASE

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Introduction

Chagas' disease is a predominantly rural disease transmitted by blood feeding triatomid vectors which readily invade dilapidated houses in tbe endemic areas of the American Continent. The disease affects about 10-12 million patients from South and Central America, and has been detected in most countries of this area. The internal migration is gradually increasing the importance of the disease as an urban problem. The social costs of Chagas' disease have not been exactly determined but since it represents the most important etiological factor of cardiac disease among people under 40 years of age, the endemy is surely imposing a heavy burden to the affected countries. This cost is represented by a significant increase of mortality rates, early retirements, hospital expenditures, unemployment and work discrimination against infected people.

The disease is usually transmitted through faeces contaminated by trypomastigote infective stages which are eliminated by the vectors after the blood feeding. The flagellates can then penetrate the vertebrate host through skin lesions or normal eye mucous membranes. Besides vector transmission, blood transfusion is now the second most important mechanism of transmission of the disease. Surveys carried out in different countries of the endemic area detected high rates of positive serology among blood donors (review: Dias, 1979). Retrospective studies de-

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monstrated that about 20% of the transfusions of positive blood induce Chagas' disease in the receptors. There has been estimated that about 10,000-20,000 new cases occur each year in Brazil as a consequence of blood transfusion. Congenital transmission is becoming increasingly important in certain endemic areas and more than 100 published cases are known, probably a small fraction of the cases occurring in nature.

In the vertebrate host the amastigote stages multiply in the cells, differentiate into trypomastigotes that burst out into the blood and again repeat the 4-5 days intracellular cycle. The acute phase of the disease which lasts 1 to 2 months is characterized by patent parasitemia, fever, oedema, signs of portal of entry and reversible myocarditis.

Meningoencephalytis may occur in children and together with cardiac lesions is responsible for 5-10% lethality at this stage. The acute phase usually subsides spontaneously and a long-life chronic phase emerges, which is characterized by a subpatent parasitemia only detectable by parasitological methods such as xenodiagnosis and hemoculture. The chronic infection may present the following clinical forms:

A - Indetermined form: most chagasic patients are asymptomatic as evaluated by current clinical methods such as electrocardiography, X-ray and even monitorized ergometry. There is strong evidence, however, that even in those asymptomatic cases minor damage of cardiac cells and myocarditis are present (Lopes *et al.*, 1981).

B - Cardiac form: a wide range of clinical manifestations, from mild electrocardiographic changes to sudden death or progressive cardiac failure, occur in that clinical form. It affects 20 to 30% of the cases, mostly young people between 20-40 years old.

C - Digestive form: pathological dilatations of the esophagus (megaesophagus) or colon (megacolon) are found in a number of cases, chiefly in certain areas as of Central Brazil. Patients with the digestive form account for about 10% of the infected people.

Association of cardiac and digestive forms are a common finding and electrocardiographic changes can be detected in about 50% of the patients with megaesophagus. Destruction of ganglion nervous cells from the autonomic nervous system is responsible for a number of clinical manifestations that reflect an unbalance of this system.

Pathogeny of Chagas' disease

Since the chronic myocardiopathy usually appears many years after the onset of the infection and at a stage in which parasites can hardly be detected in the bloodstream or tissues, a direct aggression of *T. cruzi* is considered unlikely to be responsible for the severe damage observed in Chagas' disease. For this reason, indirect mechanisms such as the "allergic" phenomena of early authors or the "auto-immune" reactions described in more recent papers have been often incriminated in this process. Santos-Buch & Teixeira (1974) described the presence of cross-reacting antigens between subcellular fractions of tissue-culture derived *T. cruzi* trypomastigotes and rabbit heart cells. Destruction of normal or parasitized allogeneic rabbit heart cells monolayers by limphocytes collected from rabbits infected or immunized with *T. cruzi* was considered as an evidence of a misdirected reaction of primed lymphocytes against the host cells. Further, the authors induced in rabbits a myocarditis by repeated injections of the *T. cruzi* subcellular fraction which cross-react with heart cells (Teixeira *et al.*, 1975).

An auto-immune humoral response was reported by Cossio *et al.* (1974). In 95% of patients with cardiomyopathy and 45% of asymptomatic patients an antibody was detected which reacts with endocardium, vascular structures and interstitium of striated muscle (EVI antibody). As this antibody can be absorbed by *T. cruzi* culture forms, a clear implication is that the EVI is directed against both parasite and heart cell structures. Antibodies against neuron cells (IgG) were observed in 98% of sera from chronic patients (Santos *et al.*, 1975b). Khoury *et al.* (1979) also reported the presence, in sera from chronic and acute patients, of an anti-nerve antibody which reacts with Schwann sheaths of somatic and autonomic peripheral nerves. This antibody could also be absorbed by *T. cruzi* and has been suggested to participate in the destruction of ganglion neuronal cells from heart and digestive tract.

Despite the bulk of evidence that points out the autoimmune reactions as involved in the pathogeny of Chagas' disease, there is still a great deal of controversy on this aspect. Some authors (Andrade & Andrade, 1979; Chiari *et al.*, 1980) were not able to confirm the previous findings on rabbits and in their experience those animals are not suitable models for the chagasic myocardiopathy. On the other hand, the specificity of the EVI antibodies had been recently challenged by Athanazio (1979)

who was unable to find a relationship between the EVI titer and the presence of clinical cardiac manifestations of Chagas' disease.

Mechanisms of resistance in T. cruzi *infections*

A steady balance between host and parasite exists at the chronic phase of Chagas' disease. No spontaneous cure occurs, the number of parasites in blood and tissues is kept at a very low level and no reagudization is observed unless as an effect, in certain conditions, of immunosuppression. Although challenge infections at the chronic phase are not giving origin to new outbreaks of high parasitemia, there is enough evidence to accept that the parasite population used for the reinfection can persist in the host together with the parasites of the original infection (Brener, 1967). This host-parasite balance reflects the presence of a solid resistance and mechanisms which allow the parasite to evade the host immune response.

Participation of the humoral response in the resistance against *T. cruzi* has been demonstrated by different ways. The role of antibodies was clearly confirmed by passive transfer of specific immune serum (Krettli & Brener, 1976) or immunoglobulin fractions (Castello Branco, 1978). Antibody-dependent cell mediated cytotoxicity (ADCC) has been recognized as an effector mechanism in the control of *T. cruzi* parasitism (Okabe *et al.*, 1980). Enhancement of *T. cruzi* infection was observed after suppression of the humoral immune response induced by neonatally initiated injection of anti-μ antibodies, a method which inhibits synthesis of IgM and IgG2a.

A direct association between lytic antibodies (demonstrated by complement-mediated lysis) and resistance was described by Krettli & Brener (unpublished data), a finding relevant to the problem of vaccination. Animals vaccinated with immunizing agents which provide only partial protection to a challenge infection display immunofluorescence antibodies (IFA) but not lytic antibodies (LA) whereas chronically infected animals and strongly resistant to reinfections present both IFA and LA. The authors suggest that the presence of LA may be used to monitorize the protective activity of immunizing agents in Chagas' disease.

A number of cell-mediated mechanisms and effector cells are apparently operative in the resistance to *T. cruzi.* Neonatal thymectomy increases the virulence of *T. cruzi* infection (Schmunis *et al.*, 1971) host immune response by "fabulation" (Eisen & Talan, 1977). Accordingly, the parasites are apparently able to release "papain-like" proteases which cleave the

surface bound immunoglobulins so that the Fc segment is degraded whereas the univalent Fab fragments remain attached by the binding sites to the parasite. The flagellate would be then protected from the harmful effect of intact antibody molecules. In the case of *CL* population, only Fab fragments could be detected by immunofluorescence whereas both Fab and Fc (IgM) fragments were observed in tbe membrane of *Y* strain trypomastigotes, a population highly sensitive to complement-mediated lysis and related effects of specific immunoglobulins.

Schmunis *et al.* (1978, 1980) reported that blood forms may perform "capping" in the presence of antibodies; they also described in detail, at the ultrastructural level, an antibody-induced mobility of surface antigens using as markers peroxidase-labelled anti-immunoglobulins.

Perspectives in the control of Chagas' disease

There are basically the following possible ways of controlling Chagas' disease: A) Vector control; B) Housing improvement; C) Chemotherapy; D) Vaccination.

Vector control by insecticides is still the most practical method of drastically reducing the transmission of Chagas' disease. In some areas such as São Paulo, Brazil, the adequate use of BHC, a chloride insecticide, has practically eradicated the intradomiciliary transmission of *T. cruzi* by *Triatoma infestans*, the most important vector of the disease in this area (for more details, see Rocha & Silva, 1979). Since the control of the intradomiciliary vectors may lead to reinvasion of the huts by triatomid-bugs which are usually less adapted to the human dwellings, a prolonged period of surveillance is necessary to consolidate the campaign. Housing improvement programs with the aim of building-up or repairing the dilapitated houses in order to make them unsuitable for the vectors depend on economic and social factors which have been very difficult to change in the underdeveloped endemic areas.

Although a few available drugs (5-nitrofuran and 2-nitroimidazol derivatives) can suppress or even cure acute infections, there is no chance in the near future to use chemotherapy as a tool for the control of Chagas' disease. The drugs are toxic, with low activity at the chronic stage and should be administered in long-term schedules (review: Brener, 1979). Mass treatment is still a remote possibility.

In summary, vector control is the most realistic approach to the

control of Chagas' disease and depends on political decisions of the affected countries. Vaccination will be here discussed in more detail.

Vaccination

In search of a vaccine active against *T. cruzi* some problems can be anticipated. First of all, a vaccine against Chagas' disease must confer total and absolute protection. A vaccine which merely attenuates the acute phase of the infection — a procedure possibly acceptable for some other infectious diseases — would be of questionable value in Chagas' disease. As stated before, tissue damage in Chagas' disease results from the long-standing, subpatent presence of the parasite in its host: heart impairment and "megas" are chronic sequels of American trypanosomiasis, not acute events. A mild acute phase is not necessarily followed by a mild chronic disease. "Unapparent" acute cases with very mild symptomatology which contrast with the typical clinical picture of the acute stage of the disease were detected in a field study in an area of active transmission (Teixeira, 1977). Those patients could be diagnosed only because the negative population was systematically monitorized by serology during 15 months and a number of such cases which otherwise would not be detected, turned positive within this period of observation. Then, unless a prolonged follow-up of those "unapparent" cases is performed in natural conditions, we cannot assume that a mild acute phase will prevent late severe tissue damage in Chagas' disease. Therefore, unless and until it is proven otherwise, one cannot be satisfied with a mere attenuation of the acute phase, since a few surviving parasites may cause chronic damage.

For the same reason, a vaccine producing itself an infection, however mild, would be equally unacceptable.

Also, an acceptable vaccine cannot induce auto-immune disease. As stated before, there is considerable evidence suggesting the participation of auto-immune mechanisms in the pathogenesis of Chagas' disease and the involvement of *T. cruzi* antigens in the triggering of such mechanisms. Therefore, any attempt to use *T. cruzi* antigens as vaccines has to be preceded by evaluation of their potential hazardousness as auto-immunity eliciting agents.

Finally, an acceptable vaccine against *T. cruzi*, for obvious reasons, also cannot induce immunosuppression.

In the foregoing discussion past achievements and future perspectives

will be analysed within the framework of these 4 basic requirements for an ideal vaccine:

1 - a vaccine cannot produce infection

2 - a vaccine has to confer total protection

3 - a vaccine cannot induce auto-immune aggression

4 - a vaccine cannot induce immunosuppression.

A vaccine capable of attenuating the acute phase and the chronic evolution of the disease could also be considered acceptable. However, it would only be acceptable if it is proven that such a vaccine is really effective in preventing the late chronic lesions of the disease in humans. This demonstration, in addition to taking many years, would have to face serious logistic and ethical problems.

Live vaccines

As early as 1913 (Brumpt, 1913) and thereafter, several authors have reported about the partial protection afforded against a lethal *T. cruzi* challenge by previous immunization (vaccination) of experimental animals with live, sub-lethal inocula of the same parasite (Hauschka *et al.*, 1950; Pizzi & Prager, 1952; McHardy & Elphick, 1978; Nussenzweig *et al.*, 1963; Neal & McHardy, 1979; Marr & Prike, 1967; Seah & Marsden, 1969; Garcia & Mühlpfordt, 1969; Norman & Kagan, 1960 and Kagan & Norman, 1961). Vaccination procedures have varied. Culture epimastigotes or blood trypomastigotes have optionally been used for immunization. Challenge has been generally performed with blood trypomastigotes of strains of variable virulence, either homologous or heterologous to the strain used for immunization. Size of inocula and schedules for vaccination and challenge have not been uniform. Different criteria have also been adopted in the evaluation of results. In spite of these experimental variables, however, it is generally agreed that a certain degree of protection, as judged by increased survival rates and attenuation of the acute phase, was achieved in most experiments. However, protection has never been complete. In every case, inoculated animals presented detectable parasitemias before and after challenge. It has also been shown that

trypanosomes of both, the immunizing and the challenging strain, could be recovered from experimental animals (Brener, 1967; Andrade *et al.*, 1970). Therefore, it seemed quite clear that infection was always present in inoculated animals and that, consequently, the protection obtained was concomitant to infection (premunition). Thus, no claims were ever made about the eventual vaccinating utility of these live vaccines, which ohviously do not meet a hasic requirement for an acceptable vaccine.

However, a "high degree protection" against virulent *T. cruzi* (various strains) was reported by Menezes (1968, 1969, 1969b) after inoculation of mice, dogs and monkeys with an attenuated *PF* strain of *T. cruzi.* Such claim was made in spite of the death of 10% and parasitemia in 17% of the vaccinated mice after challenge (Menezes, 1968). Menezes (1970, 1970b, 1971) also presented evidence for the non-infectivity of the attenuated strain and proceeded to inoculate humans with culture forms of the *PF* strain. Unfortunately, the non-infectivity of the attenuated strain was not confirmed by others. Chiari *et al.* (1973) and Chiari (1974) showed the infectivity in mice of the *PF* strain and related this infectivity to the presence of metacyclic trypomastigotes in the cultures, a variable which is difficult to control (Camargo, 1964).

A distinct approach concerning live vaccines was attempted using non-pathogenic trypanosomes of rodents prior to challenge with virulent *T. cruzi* (Kloetzel & Deane, 1971; Norman & Kagan, 1960; Deane & Kloetzel, 1974): no protection was achieved.

Souza & Roitman (1971), Souza (1974) and Souza *et al.* (1974) inoculated mice with non-pathogenic insect trypanosomatids (*Herpethomonas samuelpessoai*), obtaining partial protection against a lethal challenge of blood forms of *T. cruzi.* The investigators have also shown that sera of the inoculated animals, prior to challenge, cross-reacted with *T. cruzi* antigens in various serological methods for the diagnosis of Chagas' disease. This reported cross-reactivity between *T. cruzi* and insect trypanosomatids (see also Santos, 1973; Berrios & Zeledon, 1960; Lopes *et al.*, 1981b) might stimulate testing of non-infective trypanosomatids as vaccinating agents. Unfortunately, however, the protection obtained so far has been only partial whereas the possible elicitation of auto-immunity or immunosuppression by these vaccines has not been investigated.

Evaluation of the protection conferred by live vaccines, infective or not, has been biased by the criteria adopted for interpretation of data. Results are expressed mainly in terms of survival rates and parasitemia

levels, phenomena which are more related to the acute phase of infection. Little attention has been paid to chronic tissue damage or disease evolution. Had these aspects been taken into consideration, the interpretation of the results might have been considerably different. Thus, Brasomhrio *et al.* (1980) revealed that mice immunized with culture forms of an attenuated (TCC) strain and later challenged with virulent *T. cruzi*, developed less severe muscular lesions than non-vaccinated controls. In another line of investigation, Meckert & Laguens (1980) have shown that repeated sub-lethal inoculations in mice are more damaging, in terms of chronic lesions, than a single sub-lethal doses of the same virulent strain.

Live, non-proliferating, vaccines

The inconvenience of live vaccines lies in the preserved capacity of the immunizing inocula to proliferate and thus infect the recipient animals. This could be overcome if organisms, although alive, were rendered unable to multiply. Collier (1931) first tried trypaflavin, without success, as an attenuating agent. Other procedures have since been tested with the same purpose. Fernandes *et al.* (1965) and Hungerer *et al.* (1976) abolished the proliferative ability of culture forms of *T. cruzi* by using chemicals interfering with the synthesis of nucleic acids, Actinomycin D and Ethidium Bromide. Cultures so treated were non-infective in mice (Fernandes *et al.*, 1965; Hungerer *et al.*, 1976) or cell cultures (Hungerer *et al.*, 1976). Inoculated in mice, they afforded considerable protection against challenge with a virulent strain, as judged by the absence of mortality for the duration of the experiments. However, parasitemia, whenever investigated (Fernandes *et al.*, 1965), was detected after challenge in the animals immunized with Actinomycin D-treated flagellates. The ethidium bromide attenuated vaccine was tested in mice and displayed only partial protection (Brener, unpublished results). An interesting approach was used by Lauria *et al.* (1979) to evaluate the efficacy of this vaccine. For a period of 14 months, 41 normal and 49 vaccinated dogs were maintained in an endemic area with active transmission and then examined for Chagas' disease. From the 14 which acquired the infection, 8 were vaccinated and 6 controls.

Irradiation of culture forms of *T. cruzi* has also been tested. The initial observations of Chiari *et al.* (1968), later extended by others, have

shown that organisms irradiated with 90 kR are non-infective to mice (Salata *et al.*, 1973; Hanson *et al.*, 1973), dogs (Tomlinson *et al.*, 1980) or cell cultures (Hanson *et al.*, 1973), this strictly meeting a basic requirement for an acceptable vaccine. However, results on the immunizing properties of these preparations differed. Chiari *et al.* (1968) failed to achieve protection against a virulent challenge, after inoculation of mice with irradiated culture or flood forms. These results were in agreement with those of Salata *et al.* (1973). Contrarily, Hanson *et al.* (1976) obtained remarkable survival rates by vaccination of mice with irradiated trypomastigotes from cell cultures. Similar attempts performed in dogs were unsuccessful (Tomlinson *et al.*, 1980).

In conclusion it seems that vaccines of live, non-proliferating organisms, are unable to totally prevent infection and therefore do not meet a basic requirement for an acceptable vaccine. The auto-immunizing or immunosuppressing properties of these vaccines are also unknown.

Vaccines of killed intact organisms

Several investigators have tested the protective value of killed whole trypanosomes as immunizing agents. Muniz *et al.* (1946) reported that merthiolate-killed culture forms of *T. cruzi* inoculated into *Rhesus* monkeys were unsuccessful in protecting the animals against challenge with blood forms of the parasite. Other investigators have reported on different ways of killing, yet preserving trypanosomes intact, for vaccination purposes (Hauschka *et al.*, 1950; Menezes, 1965; Neal & Johnson, 1977; Kagan & Norman, 1961; McHardy & Elphick 1978, Kierszenbaum & Budzko, 1975). We are also aware of many unpublished experiments using heat-inactivated, formalin-killed or otherwise fixed trypanosomes in vaccination attempts. In every case, immunizing preparations were found to be non-infective in recipient animals but did not confer protection either. At best, only partial protection has been obtained after challenge with homologous or heterologous strains of *T. cruzi*. Somewhat better results have been reported by Neal & Johnson (1977) who used adjuvants to formalin-killed organisms and by Kierszenbaum & Budzko (1975) and Kierszenbaum & Ferraresi (1979) who used chaotropic ions as killing agents. In both cases, mortality rates were null or far lower in immunized mice than in controls. However, parasitemia was often detected and infection would probably be always detected in

most animals if tests more discriminatory than parasitemia had been used. McHardy & Elphick (1978) compared the effectiveness of vaccines prepared in different ways, concluding that killed vaccines are not better than vaccines made of living epimastigotes or of freeze-thawed preparations.

Very little is known concerning the auto-immunizing properties of killed whole preparations of *T. cruzi*. The only observation available is that of Muniz & Pena Azevedo (1947) who refer to myocardial lesions in vaccinated unchallenged monkeys.

Vaccines of cell homogenates and subcellular fractions

Since killed but intact as well as live but non-proliferating organisms have failed to confer protection to recipient animals, trials have been made with *T. cruzi* homogenates hoping that masked antigens could thus be exposed and rendered active for vaccination purposes.

Various ways for disrupting cells and obtaining subcellular fractions for immunization experiments have been tested. Blood or culture form of flagellates have been disrupted by freeze-thawing, low-pressure homogeneization, osmotic shock, liophylization-grinding, sonication, etc. Resulting homogenates or cell fractions obtained by differential centrifugation have, thereafter, been tested for their immunizing capacity. Unfortunately, tests for ascertaining the specific sterility for *T. cruzi* of the immunizing preparations were not always carried out. Parasitemia was seldom investigated before challenge and only in a few instances more sensitive tests for detecting vaccine-produced infection were performed (xenodiagnosis, blood cultures, inoculation into baby mice or into cell cultures). This, however, should have been routinely done since it is common knowledge that most homogenizing procedures do not kill all organisms in a preparation. Therefore, a few remaining flagellates could promote subpatent infection and thus confer concomitant protection as live vaccines do.

Johnson *et al.* (1963) used adjuvants to freeze-thawed blood forms of *T. cruzi* obtaining very poor protection to challenge with a virulent strain. Goble *et al.* (1964) reported preliminary results on the partial protection obtained by using variously disrupted culture forms *T. cruzi*. Gonzales-Cappa *et al.* (1968) reported high survival rates after challenge of mice previously inoculated with culture forms disrupted by low-pres-

sure: parasitemia although transient and lower than in controls was nonetheless detectable in immunized animals. Kaneda (1973) reported parasitemia after challenge in surviving mice vaccinated with freeze-thawed culture forms. McHardy (1977), McHardy & Elphick (1978, 1980) and Neal & McHardy (1979) thoroughly studied the protective value of freeze-thawed preparations of various strains of *T. cruzi.* McHardy (1977) also compared the effectiveness of administration routes, size of inocula, the use of adjuvants or of killing agents and the type of strain used for vaccination or challenge. Differences were found amongst the various procedures but, in every instance, parasitemia was detected after challenge, whereas mortality rates, although lower than in controls and different for the various procedures, were nevertheless significant.

Reports on immunization attempts with subcellular fractions of culture forms of *T. cruzi* are also available. Segura *et al.* (1977) obtained increased survival rates after challenge of mice immunized with a flagellar fraction of *T. cruzi* epimastigotes, although poorer results were obtained with other cell fractions. Unfortunately, no data have been presented concerning parasitemia or other means of detecting infection after challenge. Leon *et al.* (1980) immunized mice with polyribosomes of *T. cruzi* epimastigotes. After challenge with blood trypomastigotes, the authors reported increased survival rates but also detectable parasitemia in vaccinated animals. Laguens *et al.* (1980) reported attenuation of chronic damage in challenged animals which had been previously immunized with subcellular fractions of *T. cruzi.*

Cell homogenates or subcellular fractions of rodent or non-pathogenic insect trypanosomatids have also been used in vaccination attempts. Cell homogenates (Souza, 1974; Souza *et al.*, 1974; Hauschka *et al.*, 1950; Johnson *et al.*, 1963; Lara, 1972) failed to afford significant protection. Lack of protection was also reported by use of a ribosomal fraction (Grynberg *et al.*, 1976, 1977) but increased survival rates were obtained with a flagellar fraction of insect trypanosomatids as the immunizing agent (Pereira *et al.*, 1977).

It is very difficult to assess comparatively the efficacy of the various immunization procedures reported. Authors have used different experimental procedures *plus* varied criteria for data evaluation and it is known that many variables may interfere with the degree of protection achieved (McHardy, 1978). In general, it can be said that immunization with cell homogenates or fractions, always confers a certain degree of protection, as judged by attenuation of the acute phase and increased

survival rates. The protection, however, is not sterilizing. Whenever investigated, infection was detected after challenge. Therefore, these preparations are devoid of practical utility for vaccination purposes. Moreover, inoculation of mice (Segura *et al.*, 1980) or rabbits (Teixeira *et al.*, 1975) with extracts or fractions of *T. cruzi* seemed to elicit autoimmune aggression, which further precludes the practical utilization of these preparations in vaccination.

In spite of that, however, much insight has been gained on the host's humoral and cellular immune response by inoculation of animals with extracts of trypanosomes (for reviews see Teixeira, 1977; Brener, 1980; Krettli, 1980).

Vaccines of purified antigens

Reports on vaccination attempts using antigens chemically purified or partially purified are surprisingly rare.

Seneca & Peer (1966) and Seneca *et al.* (1966) used as vaccinating agent a presumed lipopolysaccharide fraction ("chagastoxin") obtained by phenol extraction of culture forms of *T. cruzi.* Mice immunized with "chagastoxin" survived longer than controls to a challenge by virulent *T. cruzi.* No data concerning parasitemia was given but since vaccinated mice also died it can be assumed that infection occurred in all animals. The authors also reported that "chagastoxin", administered simultaneously or after challenge, enhanced rather than attenuated the ensuing infection. Seneca (1969) later reported on some aspects of "chagastoxin" toxicity in mice. Kierszenbaum & Budzko (1973) tested in mice a similarly prepared lipopolysaccharide fraction (TCLP) from *T. cruzi* culture forms. Mice receiving TCLP at any time before or after challenge developed higher parasitemias and died sooner than controls. These authors called attention to the fact that the presence of TCLP in preparations of *T. cruzi* might be a risk factor in vaccination attempts.

Scott & Snary (1979) studied the protective value of a surface glycoprotein isolated from *T. cruzi* epimastigotes by affinity chromatography. This glycoprotein injected alone in mice failed to confer protection but its injection associated to adjuvants determined lower parasitemias and allowed survival of the vaccinated mice for the duration of the experiments (39 days). Controls receiving only a lethal challenge of blood trypomastigotes died within 25 days. Parasitemia, although lower and

transient, was nonetheless detected in vaccinated animals. Therefore, the protection achieved was not sterilizing. In this respect, these results are not significantly better than those obtained by the use of live or otherwise prepared vaccines or by the use of non-specific immuno-stimulants (Kierszenbaum & Ferraresi, 1979). However, these results differ considerably from those of the preceding sections because the vaccination attempt was made with a purified, chemically defined substance. Moreover, it has also been evidenced that this glycoprotein antigen does not cross-react with tissue-directed antibodies from Chagas' disease patients. These results raise the hope that vaccines of purified antigens may at last meet two basic requirements for an acceptable vaccine: to be non-infective and not to induce auto-immune aggression. It remains to be seen whether, in the future, vaccines of this kind will be able to confer total protection.

Perspectives

Recent developments in immunological methodology have considerably reduced the guesswork required in the past for the identification and production of antigens for vaccination purposes. Cell surface components can be isotopically labelled and then processed by immunoprecipitation with immune sera for identification of specific antigens. Hybridoma-produced monoclonal antibodies may further improve antigen identification while allowing the characterization, by passive immunization experiments, of those antigens capable of eliciting protective humoral response. Work on Malaria (see chapter) illustrates the usefulness and potentialities of this new methodology towards the production of an anti-parasite vaccine.

In a later stage, through recombinant-DNA technology, the desired antigens can be produced by recipient bacteria in large scale. Obviously this is not a short nor smooth road. Skill and ingenuity will be required at every step for the solution of unexpected problems. For instance, if the selected antigen is found to be a glycoprotein with the antigenic determinants at the glycosidic moiety, its production by transformed bacteria may pose serious additional problems. However, the present state of the art and its predictable future achievements, allow us to be optimistic about the outcome of the overall enterprise.

Simultaneously with the implementation of this multidisciplinary

methodology, specific problems of a Chagas' disease vaccine, have also to be worked out.

Basic research is still needed for the complete understanding of the host cellular and humoral immune response in Chagas' disease.

Concerning the antigens of *T. cruzi*, some problems also require clarifying. Surface antigens are natural candidates for eliciting protective humoral response. So, it is reasonable that these antigens be investigated first. Protective antibodies raised against surface antigens may act either by favoring the immunologically-mediated destruction of the parasite or by preventing the parasite from entering the host's tissue cells. In order to prevent infection and/or curb its dissemination, these antibodies have to be directed against metacyclic and/or blood trypomastigotes. Therefore, trypomastigotes should be used as antigen sources. However, blood trypomastigotes and metacyclic forms from the insect vector are very difficult to obtain on a workable scale. For this reason, epimastigotes, which can be easily cultured, have been preferred as antigen source. Although there is some evidence that epi and trypomastigotes share common antigens (Fruit *et al.*, 1978; Gottlieb, 1978; Kloetzel *et al.*, 1975; Snary & Hudson, 1979; Nogueira *et al.*, 1981) there is no evidence whatsoever that these common antigens are the ones capable of eliciting protection. In fact, an epimastigote glycoprotein elicited only a limited protection against a trypomastigote challenge (Scott & Snary, 1979). Thus, alternative sources for epimastigote antigens have to be tested. It is possible that trypomastigotes raised in cell cultures and that metacyclic obtained in modified culture media for epimastigotes may substitute for their hardly obtainable natural counterparts. But that has not been determined yet.

An additional problem concerns the heterogeneity of *T. cruzi*. As stated before, the existence of distinct strains of *T. cruzi* can be evidenced by various criteria. These strains differ by some biological and biochemical characteristics and may also differ from immunological standpoints.

It has been shown that various strains of *T. cruzi* share common antigens but it has also been shown that some antigens are restricted to a few strains (Nussenzweig *et al.*, 1963b; Nussenzweig & Goble, 1966; Gonzales-Cappa & Kagan, 1969).

Strains of *T. cruzi* may also differ as to their ability to evade host immune response. It has been shown that trypomastigotes of the *Y* and *CL* strains behave differently in an antibody-mediated cell-lysis test (Krettli *et al.*, 1979). It has also been shown, as mentioned, that trypomastigotes

of the *CL* but not of the *Y* strain are capable of getting rid of surface-bound antibodies by a special mechanism (fabulation) involving splicing of the antibody molecule (Krettli *et al.*, 1980).

Further complexity arises from the fact that even well-defined strains of *T. cruzi* are not entirely homogeneous. Clones of *Y* and *CL* strains have been separated in two distinct types by restriction-endonuclease digestion of k-DNA (Morel *et al.*, 1980) and also by their ability to produce metacyclic trypomastigotes in culture. Moreover, it has been known since Carlos Chagas' initial reports on *T. cruzi* that blood trypomastigotes display noticeable polymorphism. Larger and slender blood forms co-exist in the same strain. Their relative proportion, however, varies from strain to strain (Brener, 1969). It has been conjectured that some of the properties of certain strains can be attributed to the predominance of one of the other of these blood forms (Brener, 1977). Therefore, it is conceivable that these forms may also present different antigens or different mechanisms for evading the immune response, which further complicates the strategy of obtaining an effective vaccine.

So many intervening variables may generate a certain skepticism as to the eventual production of a vaccine against Chagas' disease. However, some of the problems raised above may very well be more academic than practical and do not necessarily have to be solved before an effective vaccine is achieved. Therefore, it is not implausible that a vaccine made of pure and specific *T. cruzi* antigens will be available in a near future.

Paradoxically, however, if such a vaccine were produced right now, great problems would still lie in the path for its utilization. This is so because a suitable animal model for the testing of such a vaccine is still lacking. As stated before, an acceptable vaccine for Chagas' disease cannot cause infection, immunosuppression or auto-immune aggression and also has to confer total protection. The last two requirements can only be tested in an animal model for chronic Chagas' disease that would produce lesions resembling those found in humans. Basically, this host should fulfill the following requirements: present a long-life subpatent parasitemia detectable by xenodiagnosis and hemoculture; give origin to specific antibodies which will allow diagnosis by conventional serology; induce auto-immune response; present histopathological lesions typical of chronic Chagas' disease which develop in a seasonable period of time; be available at fairly prices.

For many reasons a number of susceptible animals such as mice, rats and guinea-pigs cannot be considered as suitable chronic hosts. *Rhesus*

monkeys are apparently good models but present some shortcomings such as high prices, increasingly difficult availability and undefined time lapse between acute infection and onset of chronic lesions. *T. cruzi* acute infections in young dogs produce severe manifestations as myocarditis, high parasitemia and lethality. Chronic infections, however, have irregular and unpredictable course and only a small percentage of the infected animals show myocardiopathy; auto-immune reactions are not known. Finally, Teixeira and his group, in a comprehensive series of papers, described in rabbits infected by *T. cruzi* or immunized with *T. cruzi* subcellular fractions, manifestations characteristic of the chronic chagasic myocardiopathy. Unfortunately, those findings had not been confirmed by other groups (Andrade & Andrade, 1979; Chiari *et al.*, 1980). Ramirez & Brener (unpublished results) observed typical acute phase in rabbits infected with bloodstream and vector-derived *T. cruzi* trypomastigotes. At the early chronic stage, the animals displayed positive serological tests, positive xenodiagnosis and incipient myocardial lesions. The use of this animal model is, therefore, still controversial and more experience should be accumulated.

Finally, we should consider that even passing through the experimental screen of efficacy (total protection) and safety (absence of auto-immune reactions), a presumptive vaccine will have to face serious decisions related to the logistics of its use in humans. Pre-clinical trials in volunteers will be necessary to ascertain that it is really innocuous to humans. Then, since Chagas' disease is an endemic disease, field trials to evaluate efficacy should be carried out in rather long periods of time. Moreover, this trial would have to solve the ethical problem of keeping vaccinated and controls exposed to the hazards of infection in an area of active transmission. Nevertheless, those apparently dismaying considerations have not the intention of discouraging investigation on vaccination in Chagas' disease. If we envisage malaria, it is quite clear that some groups of scientists have to adopt a prospective view and try to anticipate events that can in the future limit the prophylaxis of Chagas' disease by vector control or housing improvement.

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CONCLUSIONS

a) *Rationale for Development of Vaccines*

With a national commitment to achieve control of parasitic diseases, presently available tools and strategies for control are effective but have limitations whicb require further field and laboratory research and constant surveillance in the endemic areas. Even when applied, the repeated failure to achieve control has been due to insufficient national resources and failure to implement sustained efforts. Development of vaccines for parasitic diseases offers potential for protection of the affected populations which could be attained within national capabilities. Recent biological technological developments applied to parasitic diseases show encouraging results toward achieving this goal.

b) *Characteristics of Vaccines for Parasitic Diseases*

The epidemiological characteristics of the parasitic diseases will influence the type of vaccine to be developed. A vaccine may create a sterilizing immunity by eliminating the existing infection and providing protection against new infection. Another type of vaccine may provide only protective immunity with some beneficial effect on the existing infection. A third type of vaccine may desensitize the immunized person so that the existing or new infections do not cause pathological changes.

c) *Evaluation of Safety of the Vaccine*

Prior to administration of a vaccine for parasitic diseases to human populations thorough evaluation of their safety by national regulatory agencies will be required. The effect of the vaccine on existing parasitic infections, the occurrence of cross reactions with host tissues and the degree of protective immunity are among the aspects to be carefully investigated, before field trials can he ethically undertaken.

d) *Evaluation of Efficacy of Vaccines for Parasitic Diseases*

The design of field trials and evaluation of the efficacy of a new vaccine will require quantitative base-line demographic, parasitological, immunological and morbidity data to accurately assess the efficacy of the vaccine in human populations and to monitor other possible unforeseen effects. Close international cooperation will be required to undertake the field trials in endemic areas.

II. Recommendations for Specific Parasitic Diseases

a) *Malaria*

Malaria is the most widespread among the parasitic diseases causing very high mortality and morbidity rates. Presently the incidence of malaria is increasing alarmingly in a number of developing countries in spite of the availability of control methods such as insecticide application and drug treatment, which were successfully applied in a number of areas. This increase in malaria, which frequently occurs in the phase of agricultural and economic development, is due to the non-existence or inadequate application of the available control measures and is aggravated by the spread of insecticides as well as drug resistance. The development of a malaria vaccine, combined with the available control measures, would contribute significantly towards the achievement of malaria control. The potential of this approach is supported by the clear evidence of natural resistance to malaria, which, however, takes many years to develop in the inhabitants of the endemic areas. Within the past few years considerable progress has been made in the research aimed at developing a malaria vaccine and potential for further advance towards this goal is greater than at any time in the past. Complete protection against malaria has been obtained upon vaccination of laboratory animals.

Three types of vaccine based on the use of different development stages of malaria parasites have experimentally been shown to have protective value. They are based on the use of the infective mosquito stage, namely sporozoites, and on the use of free asexual blood stages and gametocytes as immunogens (sexual stages). They induce very diverse specific immune mechanisms and possibly will satisfy different needs besides having different shortcomings. Attenuated sporozoites do not require the use of adjuvants, induce complete protection but their protective effect

is rather short-lived. The merozoite vaccine at the present stage requires the use of rather toxic adjuvants in order to be effective and results primarily in considerable diminution of disease manifestation of malaria. Finally, the gamete vaccine has a rather peculiar prophylactic effect by interrupting malaria transmission without affecting the course of the disease in the vaccinated individual. However, the recently developed successful approach of identifying and characterizing specific protective parasites components for their eventual use as immunizing agents might change some of the presently recognized characteristics of these potential vaccines and reduce the potential undesirable side effects.

The past inability to obtain sufficient amounts of parasites or their components, for the development of a vaccine, might now be overcome by improved culture methods, the recently developed methods of genetic engineering combined with hybridoma technologies and possibly even by chemical synthesis. The application of this technology to malaria research is also leading to the development of more sensitive methods to measure malaria incidence and monitor the protective effects of immunization procedures. Broad international support of this research effort as well as of other control methods, combined with the creation of adequate conditions for the future administration of a vaccine are essential for achieving malaria control on a world-wide basis.

b) *Schistosomiasis*

Schistosomiasis is one of the most common parasitic infections in the world. Over 600 million persons in the developing countries are exposed to the risk of Schistosome infection and over 200 million persons are currently estimated to be infected. The prevalence of this infection is increasing rapidly within endemic areas where water resources for agricultural and economic purposes are being developed.

After a developmental cycle in snails in water bodies of endemic areas, *Schistosoma* parasites infect man by penetrating the skin. Some species migrate to the blood vessels of the liver, and one species goes to the blood vessels around the bladder. In infections of long duration, the former parasites cause irreversible liver damage and the latter damage the urinary tract, particularly in children.

Presently available control tactics may be effective in most endemic areas, particularly by administration of new safe chemotherapy, to reduce prevalence, intensity of infection and the risk of development of disease.

However, a vaccine administered during early childhood and conferring long lasting protection would be a more effective method of control and should remain the ultimate goal.

Current and past research has indicated that resistance to reinfection in schistosomiasis can be demonstrated in experimental animal models and the mechanisms of induction and expression of these forms of resistance are currently being investigated. Immune responses against schistosomes can be demonstrated in man but their relative roles in regard to protective immunity or to immunopathology are still unclear.

Experimental situations which have led to the induction of protective immunity include an active infection, immunization with attenuated (irradiated) cercariae (the skin penetrating stage of the parasite) and, in a few instances, immunization with non-living parasite-derived preparations. The resistance that develops during an active infection is closely associated with the disease process and is unlikely to provide a useful approach to vaccination. The attenuated live vaccine induces a high level of protection and holds promise for the elucidation of protective mechanisms. However, potential difficulties in production and logistical supply would seem to preclude large-scale use of this methodology. The use of non-living parasite preparations as a vaccine, while yet unproven, remains a highly desirable goal.

The development of a vaccine from non-living parasite material will involve the identification of the immunizing agent, the determination of its structure, its synthesis, and the most efficacious modes of administration. It is anticipated that the development of such a vaccine will be greatly facilitated by the recent advances in biological technology, e.g., the production of monoclonal antibodies, peptide sequencing and synthesis, and recombinant DNA techniques.

c) *Leishmaniasis*

Leishmaniasis is a complex of diseases that can take different forms and are of worldwide importance. The relative toxicity of the drugs used to treat this condition, the resistance of certain forms of the disease to any kind of treatment, as well as difficulties inherent in vector control, render the search for vaccines a task of the highest priority.

Certain types of leishmanial infections, particularly the cutaneous disease known as the *Oriental sore*, are self-contained and relatively benign: the only sequellae usually consist of unsightly scars. Vaccination against

this infection is currently practiced in certain parts of the world by deliberately inoculating virulent parasites into unconspicuous sites, in order to protect against the disfiguring consequences of the naturally contracted disease. An effective vaccine other than the virulent parasite itself would undoubtedly be preferable.

Still far more important, however, is the requirement for vaccine development against the more severe forms of leishmaniasis, such as the visceral and the mucocutaneous infections. With respect to the former, it is almost certain that a degree of immunity develops in populations of endemic areas, presumably as a result of subclinical infections contracted naturally by exposure to parasite strains of low pathogenicity. In addition, clinical evidence suggests that many people recovered from the visceral infection by an appropriate course of therapy, are thereafter strongly immune against reinfection. The observation that immunity against visceral leishmaniasis does develop in these two situations makes it highly probable that a successful vaccine can be devised to protect against this condition.

Equally important is the search for prophylactic measures against mucocutaneous leishmaniasis. In this case also, the finding that immunity against the causative agent develops in certain individuals, is a strong indication that appropriate vaccination procedures can be devised. In addition, since an effective vector control is at present unforeseeable, such vaccine can be considered as the most effective way of controlling dissemination of the disease.

There are indications that dead vaccines may not be able to protect against visceral or mucocutaneous leishmaniasis. In view of the high morbidity and mortality associated with these two conditions, the use of a live vaccine that would produce a self-limited infection, even of some duration, confined to the skin, might be considered.

Whereas every effort should be undertaken to achieve this goal, great care has yet to be taken concerning the risks inherent in the permanent exposure of certain populations to the infection. In the case of cutaneous and mucocutaneous leishmaniasis of the South American continent, this refers particularly to new areas of settlement within forest regions. In this respect, it is recommended that migration programs, in countries where they exist, be accompanied and followed by sanitary measures aimed to guarantee proper surveillance and treatment of migrating populations.

d) *African Trypanosomiasis*

African trypanosomiasis is transmitted by tsetse flies and is usually

a fatal disease in both man and domestic animals. Trypanosomiasis is a major barrier to stock rearing in Africa and is to a large extent responsible for the dietary protein shortage of man in that continent. Development of an effective vaccine against animal trypanosomiasis would therefore lead to substantial improvements in human health in an area, south of the Sahara, that corresponds in size to the United States of America.

The trypanosomas that cause human sleeping sickness can also live in cattle and game animals, but are confined to natural foci. The disease is contained in these foci by regular surveillance of the population by drug treatment of those at risk, and by anti-vector campaigns. When these measures break down, however, the disease may become epidemic and cause heavy mortality. This happens frequently following climatic changes, such as draught, and as a result of population movement. An increasing number of outbreaks have occurred in recent years, notably in Sudan, Cameroon, Angola and Uganda. These epidemics are difficult to control, and it is for such control that a vaccine is needed.

A major problem in developing a vaccine against both human and animal African trypanosomiasis has been the ability of the trypanosome to change its surface antigens and avoid the host's immune response. The repertoire of such changes for an individual trypanosome is likely to be large — amounting possibly to over 100 antigenic types. As it is likely that more than one serodeme or antigenic repertoire is circulating in a given endemic focus the number of antigenic types to be vaccinated against could be enormous.

One possihle way of overcoming the difficulty posed by antigenic variation might be to vaccinate against only those antigenic types injected by the tsetse fly when it feeds. The metacyclic trypanosomes extruded by the vector appear to contain a restricted number of such antigenic types and immunizing potential hosts against these antigens should prevent infection. Determination of this number for each serodeme circulating in different foci would be a prerequisite for such a vaccine. The technology is now available, however, for filling these gaps in our knowledge, for producing the relevant antigens in bulk and for rapid identification of serodemes. Although it would be useless to pretend that a vaccine against sleeping sickness could he available within the next few years, research over this period should enable us to decide whether vaccination along the lines above will he feasible.

e) *Chagas' Disease*

Chagas' disease is closely related to the poor housing conditions prevailing in the endemic areas of the American Continent. Control of the disease depends upon the elimination of insect vectors which colonize human dwellings or such conditions and measures leading to housing improvement. Insecticide application drastically reduces intradomiciliary transmission and prevalence of the disease. It is therefore recommended that this method should be extended to all endemic areas. Housing improvement depends on social economic development. Transmission by blood transfusion is becoming an increasing hazard in endemic areas, and has both practical and ethical implications. It may be controlled by specific means such as serological screening of donors and addition of sterilizing substances to banked blood, as well as by general measures to improve medical organization in developing countries.

The implementation of a vaccine still depends on basic research to elucidate important aspects such as efficacy and safety. The possible existence of cross-reacting antigens between trypanosoma cruzi and host cells, as well as the practical logistical and ethical problems involved in the trial of a vaccine makes this method at the time being more a subject of fundamental investigation than an immediate tool for the control of Chagas' disease. Nevertheless, since some problems may emerge in the future (such as vector resistance or other unpredictable factors which may handicap control programs), specific vaccination should be further investigated. In addition, this immunological approach may substantially contribute to increase knowledge on the immunopathology of the disease, the mechanisms of resistance and even on such practical aspects as diagnostic methods.

III. Conclusions

a) *General Evaluation of the Present Situation*

A general evaluation of the present vaccine situation in the diseases discussed reveals the existence of a considerable body of information regarding the immune responses during these parasitic diseases. It is thought that this information should and will continue to increase. This will greatly enhance the prospects for vaccine development. Currently the only suc-

cessful forms of immunization in these areas involve the experimental use of living, or attenuated, organisms. Indeed, the single vaccine now used in man is for the prevention of disfigurement in cutaneous leishmaniasis and this consists of actual localized infection by living virulent organisms.

In general it is considered much more desirable to seek the utilization of non-living materials for immunization. This is based upon considerations of safety, production, and delivery.

It is toward this goal that most current research efforts are focused. The problem of the identification and large-scale production of parasite antigens for use in vaccination can now be approached with a series of new and powerful biological tools. These include monoclonal antibodies, new techniques for peptide sequencing and synthesis, recombinant DNA, and the induction of protective immune responses *without* antigen by the use of anti-idiotype antibodies.

It appears that the most progress toward vaccination against a major parasitic infection of man has occurred in regard to malaria. Currently experimental immunity can be induced to 3 different life-cycle stages; sporozoites, merozoites, and gamefocytes. The recent development of protective monoclonal antibodies against each of these stages should allow the isolation and production of protective antigens. This general approach is being used by several laboratories. In schistosomiasis immunization with attenuated organisms has been demonstrated to be efficacious in experimental and domestic animals. These and other model systems, are expected to provide further information necessary for vaccine development.

Studies on immunity to leishmaniasis have emphasized the importance of cell-mediated immunity in the host control in all three major forms of this infection and the contribution of immunoregulatory factors in determining the occurrence of disease. Existing evidence suggests that living attenuated vaccines in addition to providing good immunization may also be the most feasible for actual employment. The development of successful immunization against African trypanosomes is greatly complicated by the remarkable ability of the parasites to adapt to the immune response of the host by varying their surface antigens. However, recent work suggests that the naturally infective (metacyclic) forms of the parasite are more genetically stable and display less antigen heterogeneity and therefore may offer appropriate targets for immune attack. Both humoral and cellular immune effector mechanisms have been implicated in acquired

resistance to the acute phase of *Trypanosoma cruzi* infection. Progress has been made on the isolation of protective antigens against this phase. Little is known about the immunological factors which lead to resistance against the chronic phase of the infection. Because of the possihility that disease may be attributed to autoimmune phenomena initiated hy the parasite, caution must be exercised in future vaccine development.

b) *Political Decisions*

Recent progress in basic immunology and parasitology has made the production of anti-parasitic vaccines a realistic scientific goal. Therefore, further research on this suhject should be encouraged and supported. However, since unforeseen problems may delay or even prevent the actual development of such vaccines, all available methods to control parasitic diseases should continue to receive proper attention. The fact that many methods availahle for the control of parasitic diseases which plague developing countries are not adequately implemented may be explained not only by social-economic factors but also because those diseases do not receive the proper priority by the governments of the affected countries. Vaccines may also have to face this problem. For this reason, whenever a vaccine would be available, it will be essential to ensure the fullest degree of approval and cooperation of country authorities with the immunizing program. It is equally essential that the population should be sufficiently educated to understand the need for vaccination and willingly agree to the procedure.

c) *International Collaboration*

International collaboration in the health field has achieved success in many instances, as for example in the control of yellow fever in Africa and South America and, most recently, in the smallpox eradication program. Support should be given to international efforts such as the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, a joint venture of over 40 developed and endemic countries to stimulate and accelerate the development of human resources and improved tools for control of parasitic disease. These efforts must be urgently multiplied to face the challenges of global population growth, increasing poverty, urbanization and malnutrition.

All nations of the developing and developed world, notwithstanding their social and political status, must cooperate to achieve effective control of parasitic disease, even overcoming national rivalries and arbitrary political barriers. Collaboration between investigators and scientific institutions in developing and developed countries in the research efforts towards vaccine development should be implemented.

Information on changing epidemiological patterns of parasitic diseases, particularly epidemics, should be communicated internationally. National pride should neither hinder the potential benefits of International Cooperation nor jeopardize the health of the affected populations.