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INTERACTION OF HEPATOCYTES WITH THE MALARIA PARASITES *PLASMODIUM FALCIPARUM*
AND *PLASMODIUM VIVAX*.

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Recent progress in the culture of liver stages of human plasmodia (1, 2) has provided many opportunities to study the interaction of sporozoites with host cells and parasitic development. *P. falciparum* sporozoites, obtained from infected mosquitoes were added to monolayers of primary human - or rat hepatocytes. Samples were fixed for immunofluorescence (IFA) and electronmicroscopy (EM) at 2 and 4 hr. and at 2, 4, 5, 7 days after inoculation. Only in human hepatocytes numerous parasites were seen at days 4, 5 and 7. However, *P. falciparum* sporozoites appeared to attach and penetrate both rat- and human hepatocytes. With EM no interiorization of sporozoites into hepatocytes has been visualized. However, after invasion the parasites were found in parasitophorous vacuoles in the primary human hepatocytes, in the frozen-thawed human hepatocytes and in rat hepatocytes. Once inside, the sporozoites showed a marked tendency to locate close to, or even inside the host nucleus. These results were similar for the three cell types involved and corroborate earlier work on *P. berghei* *in vitro* and *in vivo* (3). The sporozoites were seen to be characteristically pointed with their apical ends into the host cell which confirms previous results with *P. berghei*. Cytostomes were sometimes seen in intracellular sporozoites.

Whether these have a function during intrahepatocytic transformation and growth is unclear.

P. vivax sporozoites were added to monolayers of cryopreserved-thawed human hepatocytes. After 8, 9 and 10 days post-inoculation the cultures were prepared for IFA and EM.

In the 8 and 9 days samples, some parasites were found with IFA measuring from 15-20 μ m. No indication of hypnozoites was found with IFA. Currently this material is examined with EM.

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METHODS FOR THE DETECTION OF ANTIBODIES AGAINST MALARIA SPOROZOITES IN HUMANS.

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Sporozoites of plasmodia are transmitted in small numbers by the bite of infected mosquitoes. Exposed individuals form antibodies, which are specific for that stage and not for blood forms. Monoclonal antibodies were generated using sporozoites of *Plasmodium falciparum* that are routinely produced in Nijmegen. These antibodies against the circum-sporozoite protein also react with recently produced synthetic peptides (NANP)₄₀ and recombinant protein (R32TET32, WRAIR Washington of *P. falciparum* sporozoites. The artificial antigens were applied in direct solid phase ELISA tests for the detection of anti-sporozoite antibodies. As an existing control the indirect immuno-fluorescent antibody test (IFA) was used with glutaraldehyde-fixed sporozoites on slides.

Monoclonal antibodies against *P. falciparum* sporozoites strongly reacted in the IFA and ELISA tests, whilst those against *P. falciparum* blood forms or against sporozoites of *P. vivax* and *P. malariae* (*P. brasilianum*) did not react.

Nearly 400 healthy blood donors from Nijmegen and Geneva were tested at a 1:200 dilution. The very rare ones that did have a positive titre, also appeared to be reactive in the IFA. Discrimination between positives and negatives was made at a cut-off value of the mean plus 3 standard deviations. In smaller trials a negative blood donor serum was serially diluted along with the positive control and patient sera, and cut-off points were found by subtracting twice the negative control value at the corresponding dilution.

Two sets of 30 serum samples were tested in a double blind study. Both sets contained sera from malaria patients, individuals from endemic areas with other diseases and Caucasian blood donors. IFA and ELISA gave results that were well correlated, though the sera could be diluted much further in the ELISA (starting dilution IFA 1:20, ELISA 1:200). 43% of Caucasians with acute malaria tropica did show anti-sporozoite antibodies.

Using the two artificial antigens in ELISA tests we found virtually identical results from the reactive and non-reactive sera.

It was demonstrated with IFA and ELISA that children from endemic areas (Gabon, Kenya) show low titres if any, and that most adults have higher titres. This may reflect the cumulative effect of repeated exposures to bites with infected mosquitoes.

Application of the ELISA for anti-sporozoite antibodies in sero-epidemiological studies will prove to be crucial for the understanding of exposure frequency to sporozoites and hence give a better indication for the transmission of malaria in human populations than was possible up till now.

THE PREPARATION OF MONOCLONAL ANTIBODIES AGAINST EGGS OF *ONCHOCERCA VOLVULUS*.

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The blindfolding disease Onchocerciasis is caused by an ovoviviparous, whip-like parasite *Onchocerca volvulus*, which belongs to the filariodea family of nematodes. It is transmitted by small *Simulium* insects (Blackfly) which are night and day biters, whose breeding place is swift running water: hence the association of the disease with waters has given rise to the name "Riverblindness".

Current diagnostic methods are limited in the detection of parasites in the skin (skin snips), in the eye or the presence of suggestive clinical signed changes.

These methods are of limited use in the monitoring of vector control programs because they can not detect prepatent infections and the parasites are frequently not recognized in light infections because of sample errors. This means that there is an urgent need from the standpoint of vector control to develop a rugged qualitative field assay with good predictive value in the diagnosis of prepatent infections.

In 1984 Forsyth succeeded in making monoclonal antibodies against stage specific surface antigens of eggs of *Onchocerca gibsoni*, a bovine filarial parasite, which could be used to detect antigen in sera.

This led us to the idea to do the same experiments with eggs of the human filarial parasite *Onchocerca volvulus*.

Selection by IFAT and ELISA indicated that we succeeded in producing monoclonal antibodies against eggs of *O. volvulus* showing cross-reactivity with *Dipetalonema viteae* and *Dirofilaria immitis* but not with *Schistosoma mansoni*, *Brugia pahangi*, *Ancylostoma caninum*, *Trichinella spiralis*, *Ascaris suum*, *Echinococcus granulosus* and *Taenia crassiceps*.

Three monoclonal antibodies (all of the IgM class) showed specificity because only a reaction with *O. volvulus* was found.

All the Moab's showed recognition of antibodies on egg and uterus membranes as indicated by IFAT using acetone fixation. If Rossman fixation was used some Moab's showed a reaction with the cuticle and/or hypodermis indicating a possible role of polysaccharides in the reaction.

Weiss (1985) publishing the results of a similar experiment, did not succeed in making specific Moab's i.e. Moab's showing only a reaction with *O. volvulus*.

It is therefore our aim to characterize these specific Moab's in more detail by the reaction with antigenic bands of filarial parasites on Western blots as described before (Wauters et al: Trop. geogr. Med in press).

After that an evaluation can be made for the usefulness of these Moab's in an immunological test for *O. volvulus* infections in patients.

LABORATORY MODEL OF *DIPETALONEMA VITEAE*

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In 1952 Chabaud described a new filarial species *Dipetalonema blanci* discovered in the jird *Meriones libicus* (Lichtenstein 1823), a burrow dwelling rodent living in the open sandy plains of North Africa and Asia minor.

Later in 1957 Chabaud decided that it was identical with the parasite *Litomosa wite* (Krepkogorskaja 1933) found in *Rhombomys opimus* (Lichtenstein 1823).

From that moment the name *D. viteae* was introduced although the name *D. witei* is also used.

During epidemiological field studies in Iran, Chamsa and Weiss (1969) found that there were 3 vertebrate hosts for *D. viteae*: *Meriones libicus*, the newly discovered *M. persicus* (Blanford 1875) and *R. opimus*.

The chief vertebrate host used in our work is *M. libicus*, originally obtained from Prof.Dr. Weiss (1983) and since that time a breeding colony has been maintained in the Animal Division of this Institute (GDIA).

Although the parasite is found in the tick *Ornithodoros tartakovskyi* (Olenev 1931) and *Rhipicephalus* spec the first is considered to be the principal artropode vector.

In 1953 Baltazard established the first laboratory infection and in 1961 Worms succeeded in obtaining good results with another argasid tick *O. moubata*.

In our experiments *O. moubata* was used as the intermediar vector which we re obtained from Prof.Dr. Weiss in 1983.

Ticks (reared at 26 °C and 90% relative humidity) were successfully fed on uninfected caviae (feeding percentage of 95%): after a short bloodmeal (45 min) most tickes detach themselves from their host and 8-10 days later eggs appear laid by adult females.

The first stage, a hexapodous larva, ends without previous nutrition with a moult leading to the first nymphal stage. Five more nymphal stages will follow, each after a bloodmeal, before the adult stage is reached.

Ticks of different age were fed on infected jirds (50-150L₁ per 20 mm³ blood) and after 2 months the infective larvae were isolated using a Binocular dissection microscope and collected with a Pasteur pipette. Results showed that the early stage ticks (stage 3) contained a low number of L₃ (35L₃) but the numbers increased when older ticks were used (stage 5 60L₃; stage 6 90L₃ and stage 7 236L₃). One single tick may support as many as 1100L₃ which can remain infective and alive for at least 180 days. After subcutaneous inoculation of 50L₃ adult worms develop in all of the 40 male jirds used up till now. In cases where the infection rate was increased the mortality rate increased significantly.

Male golden hamsters (*Mesocricetus auratus*) used as additional host were inoculated with 250L₃ and in all 19 animals worms could be isolated. In contrast to jirds hamsters develop an immunorespons against L₁ but tolerate adults for up to 15 months postinfection. Our results showed no correlation between time after infection and number of isolated worms; 180 days postinfection still a great number of adults were found mainly in the subcutaneous tissues and the fascia of the muscles of the axilla, groins and between the scapulae on the dorsal side. Some worms were found on the head or the limbs but no strict rules can be given. The same holds true for the jird but worms tend to be located only in the subcutaneous tissues. Using a Binocular dissection microscope + 20 adults and + 125 adults could be isolated from respectively the jird or hamster (with 170 as largest number).

LUMINOL-DEPENDENT CHEMILUMINESCENCE OF LYMNAEA STAGNALIS HAEMOCYTES.

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In the pond snail *Lymnaea stagnalis*, intermediate host of the schistosome parasite *Trichobilharzia ocellata*, macrophage-like haemocytes (blood cells) play a key role in internal defence. The haemocytes are involved in defence reactions such as phagocytosis of e.g. bacteria and encapsulation of e.g. non-compatible (schistosome) parasites. Subsequently, the invading organisms are killed intra- or extracellularly. The mechanisms by which the haemocytes establish killing are not known, however.

An important intra- and extracellular killing mechanism used by vertebrate phagocytes involves reactive oxygen intermediates (ROI). These ROI - super-oxide (O_2^-), peroxide (H_2O_2), hydroxyl radicals (OH^\bullet) and singlet oxygen (1O_2) - are strong oxidative substances and consequently toxic for e.g. bacteria or parasites. The production of ROI during the respiratory burst is accompanied by chemiluminescence: the emission of light (photons) by the phagocytes. The intensity of emitted light is usually very low and for detection it has to be artificially enhanced by e.g. luminol. Thus, luminol-dependent chemiluminescence (LDCL) can be used to monitor ROI production of phagocytic cells.

In this study, haemocytes of adult *L. stagnalis* were incubated *in vitro* with a variety of phagocytic stimuli: phorbol myristate acetate, latex particles, heat-killed bacteria (*Escherichia coli* and *Staphylococcus saprophyticus*), zymosan (yeast) particles. The haemocytes showed different LDCL responses for each agent used. Zymosan elicited a high, long-lasting LDCL with an optimal particle-to-haemocyte ratio of 12 to 1. LDCL was partially inhibited by super-oxide dismutase indicating the involvement of superoxide anions. LDCL was strongly decreased by azide and cyanide, which is probably caused by inhibitory effects on peroxidase and singlet oxygen.

In conclusion, these results indicate that *L. stagnalis* haemocytes generate ROI when they are phagocytically stimulated with various (non)particulate agents. This ROI production can be monitored *in vitro* using LDCL. The possible role of ROI in intracellular killing of bacteria and in extracellular killing of (schistosome) parasites by snail haemocytes will be a subject of future research.

GLYCOLYTIC ENZYMES FROM *TRYPANOSOMA BRUCEI**

IDEAL TARGETS FOR DRUG DEVELOPMENT?

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Trypanosomatids are the causative agents of a number of tropical diseases like sleeping sickness, nagana, Chagas' disease, Kala-azar, oriental sore etc. Until now no satisfactory drugs exist against these parasites. In the Trypanosomatids, the first seven reactions of glycolysis take place in a special organelle, the *GLYCOSOME*, a situation which differs from mammalian cells, where the entire glycolytic pathway is localized in the cytosol. Therefore, it was assumed that the glycolytic enzymes from Trypanosomatidae would have different properties and could serve as ideal targets for drug development.

The physical, chemical and structural differences between the glycolytic enzymes from the parasites and their hosts, can best be studied on purified enzymes. We have therefore developed a purification procedure for the Trypanosomal enzymes which results in the simultaneous purification of nine glycosomal enzymes. Starting from 25 g trypanosomes (wet weight), 'cultured' in 50 Wistar rats, the following quantities of pure enzyme can be obtained (% recovery): 1.6 mg HK (15%), 2.0 mg PGI (40%), 5.8 mg PFK (41%), 13 mg ALD, 0.4 mg TIM (47%), 4.8 mg GAPDH (50%), 1.9 mg PGK (43%), 3.2 mg GPDH (36%) and 2.6 mg GK (25%).

Preliminary kinetic studies with TIM and ALD revealed no big differences with the mammalian counterparts. Trypanosomal GPDH was competitively inhibited by suramin whereas the rabbit muscle enzyme was not. Furthermore, hexokinase differs from the mammalian hexokinases in that it possesses a broad specificity towards the nucleoside triphosphates. This indicates that the binding site for this substrate must have a different structure.

An extensive study on the subunit (SDS-PAGE) and native (Gel filtration) molecular weights of trypanosomal and mammalian enzymes revealed striking differences for HK and PFK. Whereas the mammalian HK's are monomeric enzymes with a $M_r = 100$ kDa, the trypanosomal HK has a subunit $M_r = 50$ kDa and forms hexamers (300 kDa) under various conditions.

PFK of both trypanosome and mammals are tetrameric enzymes, however the subunit size is completely different (50 kDa and 80-90 kDa resp.).

CONCLUSION: Based on our present knowledge, both hexokinase and phosphofructokinase from *Trypanosoma brucei* differ from their mammalian counterparts and may therefore serve as excellent targets for drug development.

Abbreviations: HK = Hexokinase; PGI = Glucosephosphate isomerase; PFK = Phosphofructokinase; ALD = Fructose-1, 6-bisphosphate aldolase; TIM = Triosephosphate isomerase; GAPDH = Glyceraldehydophosphate dehydrogenase; PGK = Phosphoglycerate kinase; GPDH = Glycerol-phosphate dehydrogenase; GK = Glycerol kinase; SDS-PAGE = Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

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STUDIES ON THE GENES FOR GLYCOLYTIC ENZYMES OF *TRYPANOSOMA BRUCEI**.

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We have made a start with the study of the genes encoding the glycolytic enzymes of *T. brucei*. This study should inform us about the structure of the genes, their genomic organization and the regulation of their expression. The determination of the nucleotide sequence of the genes should enable us to get easily the information of the primary structure of the proteins. Knowledge of these primary structures is indispensable to develop trypanocidal drugs for which the parasite's glycolytic enzymes will form the targets.

From a recombinant library of DNA complementary to the poly A⁺ RNA from bloodstream form trypanosomes the cDNAs for glyceraldehyde-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), triosephosphate isomerase (TIM) and aldolase (ALDO) were isolated, using the corresponding gene-probes of other organisms, *i.e.* yeast and mammals. The cDNAs were subsequently used as probes to isolate genomic clones of these genes as well.

Our initial analysis of these clones and the nuclear DNA has shown that the trypanosomal genome contains two closely linked, very similar GAPDH genes. A similar organization has been found for the PGK genes for which also a truncated pseudogene seems to exist. In contrast, only one TIM and one ALDO gene have been observed. Because GAPDH and PGK occur both in a microbody-like organelle, the glycosome and in the cytosol, whereas TIM and ALDO are only found in the glycosome, our data suggest that glycosomal and cytosolic isoenzymes are encoded by separate but closely linked, very similar genes. Our analyses of the transcripts and the DNA sequencing seem to support this view.

The sequence analysis of the GAPDH, PGK, TIM and ALDO genes, as well as that of their flanking regions, is well under way. The data obtained so far, when compared with the published sequences of the genes from other organisms, indicate that the glycolytic genes are well conserved throughout evolution.

However, specific regions seem to be present in the trypanosomal genes.

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