Thursday, December 18, 2008

Weizmann Institute [Schmidt Auditorium]

09:40 – 11:10 Session 1: *Entamoeba histolytica* (Molecular pathogenesis)

Manipulation of gene expression in *Entamoeba histolytica*

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E. histolytica is not amenable to standard genetic techniques as it has no sexual cycle, the genome is polyploid and many virulence determinants are members of large gene families. Methods of homologous recombination have not yet been developed for *E. histolytica*, therefore the main way to study the particular function of a gene has been by stable episomal transfection for both the up- or down-regulation of genes of interest. Plasmids constructs, including tetracycline-inducible vectors, containing genes in the sense, anti-sense or mutated forms for dominant-negative effects, have been successfully used by a number of investigators and have significantly contributed to our understanding of the role and function of many *E. histolytica* genes in pathogenesis as well as in parasite metabolism. Recently, two additional possibilities for the suppression of gene expression have been introduced, (i) selected inhibition of gene expression by either direct administration or by episomal generation of siRNA molecules and (ii) by epigenetic gene silencing following transfection with a plasmid that contains the 5' upstream region of the amoebapore-a gene which includes a truncated segment of a repetitive SINE retroposon element that is transcribed in the opposite orientation. Each of the above mentioned techniques has advantages and disadvantages which will be discussed.

Transcriptional profiling of the amoebapore-silenced G3 trophozoites: analysis of a significantly up-regulated gene

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Changes in gene expression in *E. histolytica* as a result of silencing of the amoebapore gene were determined by transcriptional profiling of the parent (HM-1:IMSS) versus the gene silenced parasite (G3) using an Affymetrix microarray. Validation by RT-PCR of several of the numerous down- and up-regulated genes confirmed the results obtained on

the microarray. Analysis of one of the hypothetical genes (459.m00030), whose transcript was very significantly up-regulated (>200 x) in G3, using antibodies raised against a recombinant copy of the protein, failed to detect this protein either in lysates of the parent strain HM-1:IMSS or in those of the gene silenced G3 trophozoites. Trophozoites (HM-1:IMSS) transfected with a plasmid to over-express this gene also had higher levels of transcript but no detectable protein. This preliminary finding indicates that although the transcription level can serve as an indication for the resulting protein level, not always the up-regulated coding sequence examined will result in an over-expressed protein.

Chromatin modifications in epigenetically gene-silenced *Entamoeba histolytica*

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The protozoan parasite *Entamoeba histolytica* expresses a number of virulence factors that are responsible for the development of intestinal inflammation and dysenteric amoebiasis. Previous investigations focusing on the amoebapore A virulence gene (*Ehap-a*) discovered that by transfecting parasites with a plasmid containing a segment (473bp) of the 5' upstream sequence of the *Ehap-a* gene as well as a truncated segment of a neighbouring SINE1 element that is transcribed from the opposite strand, a complete transcriptional silencing of that gene was induced. This silencing does not depend on siRNA nor DNA methylation. To determine which epigenetic changes occurred, we analyzed chromatin and histone modifications. Careful digestion of the chromatin with a Micrococcal nuclease revealed a significant resistance in the promoter region of the *Ehap-a* gene in the silenced strain (named G3) as compared to the parental amoeba strain (named HM-1). Methylation of histone H3 on lysine 4 is known to be associated with transcriptional activity. We therefore analysed the presence of such a modification using a ChIP assay with an antibody recognizing the histone H3K4 both in its dimethyl- and trimethylated form. We found that the methylation level of H3K4 in the domain of the *Ehap-a* gene was significantly reduced in the silenced G3 ameoba strain as compared to the parental HM-1. All these results were successfully confirmed for other silenced virulence genes in additional silenced amoeba strains. Altogether, our data show that heterochromatin formation and histone modifications are at the basis of the gene silencing mechanism that occurred in G3 amoeba. Further experiments will be needed to decipher which factors or enzymes are initiating and propagating these epigenetic changes.

An inverzincin from *Entamoeba histolytica*

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Entamoeba histolytica expresses an array of proteolytic enzymes which may play a role in pathogenesis. One of these is a metalloprotease (EhMP16C, EC 3.4.24.-) of the inverzincin family. Its closest homologue (29 %), the Presequence Protease from *Arabidopsis thaliana* has been extensively characterized. It is responsible for the degradation of the targeting peptides after they are removed from imported proteins to mitochondria and chloroplasts by a mitochondrial processing peptidase or stromal processing peptidase, respectively. In the amoebic trophozoites, the enzyme is a soluble protein of 111 kDa and it is encoded by a single copy gene with an uninterrupted ORF of 2910 bp. The recombinant amoebic protease is able to degrade galanin, an unstructured peptide of 28 a.a. Its proteolytic activity is inhibited by EDTA, 1,10-phenanthroline and 10 mM $ZnCl_2$, all of them characteristic inhibitors of metalloproteases. In order to identify the role of this enzyme in *E. histolytica*, amoebic trophozoites HM-1:IMSS were transfected with plasmids (i) to over-express or (ii) to down-regulate the expression of this gene. Different tests are being conducted with the transfectant amoebae. The corresponding gene in *E. dispar (edmp*16C*)* is 87% identical to *ehmp*16C. Attempts to crystallize the recombinant protease from *E. histolytica* and compare its structure with the one from the non-pathogenic species are in progress.

11:30 – 12:30 Session 2: *Entamoeba histolytica* (Molecular pathogenesis)

Enolase interacts with *Entamoeba histolytica* cytosine-5 methyltransferase Dnmt2 homolog (Ehmeth) and modulates its substrates binding activity

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Evidences for DNA and RNA methyltransferases activities of cytosine-5 methyltransferases of the Dnmt2 family in different organisms have been previously reported. In contrast to Dnmt1 and Dnmt3 proteins were many interacting proteins have been identified, no interacting candidate within the Dnmt2 family has ever been reported. In an attempt to gain insights into the molecular function of Ehmeth, the *Entamoeba histolytica* cytosine-5 methyltransferases Dnmt2 homolog, we have performed a yeast two-hybrid screen and identified enolase as an Ehmeth binding protein. Enolase catalyzes the conversion of 2-phosphoglycerate (2PG) to phosphoenolpyruvate. We showed that enolase has both a cytoplasmic and a nuclear localization in the parasite. Its binding to

Ehmeth was confirmed both in vitro and in vivo by pull down experiment. In vitro evidences show that enolase modulates the ability of Ehmeth to bind SMA/R DNA and tRNA. The validity of the Ehmeth-Enolase interaction was extended to *D.melanogaster* Dnmt2 homologs. These results suggest that enolase is a protein with multiple roles in the parasite and constitute the first report of a Dnmt2 interacting protein.

From DNA methylation to DNA methylation recognition: the unique *Entamoeba histolytica* epigenetic machinery

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DNA methylation and methylated DNA target recognition represent central epigenetic events. We found that in the parasite *Entamoeba histolytica*, DNA methylation is catalyzed by Ehmeth, a DNA methyltransferase belonging to the enigmatic Dnmt2 protein family. DNA methylation occurs mainly in LINE retrotransposon, rDNA and S/MAR element. The range of Ehmeth substrates was recently expanded to tRNA raising the possibility that the biological roles of Ehmeth might be broader than controlling repetitive elements. Enolase, a glycolytic enzyme, interacts with the catalytic domain of Ehmeth and modulates its activity. Enolase represents the first example of a Dnmt2-interacting protein. In most eukaryotes, methylated cytosine recruits methylated CpG binding proteins (MBDs) that interact with histone deacetylase to alter the chromatin structure and lead to the silencing of gene expression. In *Entamoeba* no "classical" MBDs are present. EhMLBP has been identified as a protein that specifically binds to methylated LINE retrotransposons and rDNA. EhMLBP is unique to Entamoeba parasites, which makes this protein a possible target for treating amebiasis. This potential has been evaluated. Downregulation of EhMLBP using antisense technology resulted in trophozoites with impaired growth and cytopathic activity. This indicated that EhMLBP is an essential protein. As an approach to identify molecules that inhibit EhMLBP activity, a selective biopanning assay was performed using the DNA binding domain of EhMLBP and the Ph.D-12 phage display peptide library. Remarkably, four out of the eleven phages selected after three rounds of biopanning expressed the peptide "SYFDQNERWGAP" (Pept3) at their surface. The binding of EhMLBP to Pept3 was confirmed by ELISA. The growth of *E. histolytica* transfectants expressing Pept3 was significantly impaired compared to that of trophozoites expressing a scrambled version of Pept3. These results highlight EhMLBP as an essential constituent of the parasite *E. histolytica* and a novel target for anti-amebic chemotherapy. We recently extended the list of EhMLBP substrates by using an affinity-based technique in which the C-terminal DNA binding domain of EhMLBP (GST-CterEhMLBP) was used as the ligand. Bioinformatic analysis of the DNA sequences that were isolated by this affinity method revealed the presence of a 29-nucleotide consensus motif that includes a stretch of eleven adenines. Four of these sequences, namely those that encoded either dihydrouridine synthetase, RAP GTPase activating protein, serine/threonine protein kinase or a leucine-

rich repeat containing protein (LRPP) were then selected for further analysis. We established that EhMLBP binds preferentially to their methylated forms and that methylated cytosines are present in *LRPP* and to a lesser extent in the other genes. The sensitivity of EhMLBP to distamycin A and its ability to bind to the consensus motif indicate that an adenine stretch is involved in the mechanism of DNA recognition. These results reinforce the notion that this protein is an innate methylated DNA binding protein.

Expression of *Entamoeba histolytica* methylated LINE binding protein EhMLBP under heat shock

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Adaptation to environmental stress is a key process that allows the unicellular parasite *Entamoeba histolytica* to survive in its human host. We previously characterized EhMLBP as a protein essential for the growth and the virulence of the parasite. EhMLBP binds to methylated repetitive DNA and it constitutes with the DNA methyltransferase Ehmeth the core enzymes of the epigenetic machinery present in the parasite. In an effort to determine whether *E.histolytica* epigenetic components are regulated by environmental stress, we studied the expression of EhMLBP under oxidative and heat stresses. EhMLBP expression was moderately up-regulated under oxidative stress whereas under heat shock its expression was strongly induced. The functionality of the heat shock element (HSE) binding sites identified in the EhMLBP promoter region was confirmed by southwestern blot analysis and promoter mapping. EhMLBP localization in the nucleus and its binding to DNA differed between control and heat shocked trophozoites. Finally, we observed that trophozoites downregulated for EhMLBP expression were more sensitive to heat shock than control trophozoites. These results constitute the first example of an epigenetic constituent regulated by environmental stress in this organism and they emphasize the crucial role played by EhMLBP.

13:45 – 14:15 Session 3: Human Amoebiasis

The cysteine protease EhCP-A5 is essential for Entamoeba histolytica invasion and for inducing inflammatory responses in an ex-vivo human colon model

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Amoebiasis, a human intestinal infection affecting 50 million persons every year, is caused by the protozoan parasite, *Entamoeba histolytica*. To study the molecular mechanisms underlying human colon invasion by *E. histolytica*, we have set up an ex vivo human colon model that mimics the early steps of amoebiasis. Using scanning electron microscopy, histology and two photon laser microscopy live imaging analysis, we have determined that *E. histolytica* degrades the protective mucus coat during the first two hours and then detaches the enterocytes and penetrates into the lamina propria. Significant cell lysis and inflammation was marked by the secretion of the proinflammatory molecules, which were detected after four hours of incubation. *Entamoeba dispar*, a closely related non-pathogenic amoeba that also colonizes the human colon, was unable to invade colonic mucosa, to lyse cells and did not induce an inflammatory response.
The behavior of trophozoites silenced in genes coding for known virulent factors such amoebapores, the Gal/GalNAc lectin and cysteine protease 5 (CP-A5), which have major roles in cellular death, adhesion (to target cells or mucus) and degradation of the mucus, respectively, were examined as well as their tissue responses. Our data revealed the essential role of the cysteine protease EhCP-A5 in the parasite's penetration of the colonic mucosa and in the induction of the host inflammatory response. Studies are in progress to examine the impact of human inflammatory molecules on the amoeba invasive process.
This work was supported by a grant of Pasteur Institute (PTR178) and by a grant from the Pasteur-Weizmann Scientific Council.

Epidemiology and pathophysiology of amoebic liver abscesses

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The intestinal protozoan parasite *Entamoeba histolytica* is endemic in large parts of the world and considered responsible for millions of cases of invasive amoebiasis. Normally present in the large bowel, the amoebae usually persist for months or even years as asymptomatic gut infection. However, occasionally the parasite penetrates the intestinal mucosa and induces colitis or disseminates to other organs where it induces abscess formation. Amoebic liver abscess (ALA) is the most common extraintestinal manifestation of invasive amoebiasis as more than 99% of *E. histolytica*-induced abscesses are located within the liver. The disease is characterized by rapidly evolving, massive tissue destructions due to apoptotic and necrotic desintegration of hepatocytes. Interestingly, less than 10% of *E. histolytica*-infected individuals develop ALA and in contrast to intestinal amoebiasis, ALA greatly predominates in adult males (>85%) but is rare in females or children. Since humans are the only relevant host for *E. histolytica*, experimental studies concerning this sexual

dimorphism have been hampered by the lack of a suitable animal model. Recent experimental data from a mouse model of ALA have indicated that immuno-competent animals are usually resistant to *E. histolytica* infection of the liver as this infection induces an early and rapid IFN- response by natural killer T (NKT) cells, which finally leads to killing of amoebae by activated macrophages. Specific activation of NKT cells, which are present in considerable amounts within the liver, is induced in a CD1d-restricted manner by an *E. histolytica* lipopeptidoglycan (EhLPPG), located in large quantities on the surface of amoeba trophozoites. Interestingly, activation of NKT cells and production of IFN- by EhLPPG is much weaker in male versus female mice. Thus, killing of amoebae is incomlete in male mice and results in prolonged persistance of parasites within the tissue. This parasite persistance and the presence of IL-17 are required for accumulation of granulocytes and the development of large and clinically relevant liver lesions. In addition to EhLPPG-triggered innate immune mechanisms, development of ALA is influenced by sex hormones. Transfer of testosteron into female mice and challenge with *E. histolytica* trophozoites was found to produce large abscesses similar to those seen in male mice and orchiectomy of male mice revelaed small abscesses similar to those of female mice.

Molecular diagnosis of intestinal parasites in patient care and epidemiology

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Twelve years ago molecular diagnostics for the differentiation of *E. histolytica* and *E. dispar* were implemented in Leiden and proved to be highly sensitive and specific. At that time, using PCR only as an additional technique to differentiate cysts and/or trophozoites that were seen by microscopy, it was never anticipated to use PCR as a first line diagnostic. During the following years the isolation of parasitic DNA from faecal samples and PCR techniques, were improved and simplified. Moreover the introduction of real-time PCR made it possible to multiplex different targets into one reaction. These new technical possibilities made it feasible to introduce PCR also for the detection of other intestinal parasites. A multiplex real-time PCR for the simultaneous detection of the three most important diarrhoea causing protozoa and an internal control proved to be a sensitive and specific method for the detection of *E. histolytica, G. lamblia* and *Cryptosporidium* (HGC-PCR). At this moment, (multiplex) real-time PCRs are available for an extended range of parasite species. These assays have been applied in several diagnostic settings and showed an excellent performance in sensitivity and workload. For example, two studies were performed to define a diagnostic strategy for the implementation of molecular methods in the routine diagnosis of intestinal parasitic infections in general practice patients and in returning travellers and immigrants. The application of these assays in epidemiological studies offers high throughput detection and quantification of multiple parasitic targets and the possibility of additional genotyping.

15:35 – 16:50 Session 4: Intestinal parasites

Epidemiology of Giardiasis in Israel during 2001-2008

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Background: Giardiasis is a diarrheal illness caused by the parasite *Giardia intestinalis* (also known as *Giardia lamblia*). This flagellated protozoan, which is transmitted through stool of an infected person or animal (domestic and wild mammals) can survive outside the host body and in the environment for prolong periods. Giardia infection is transmitted by the fecal-oral route and results from the ingestion of Giardia cysts through the consumption of fecally contaminated food or water or through person-to-person or animal-to-person transmission. During the past 2 decades, *Giardia* has become recognized as one of the most common causes of waterborne disease (drinking and recreational) in humans in the United States. In Israel we are witnessing an emergence of this very contagious zoonotic disease during the last decade. Giardiasis is a mandatory reportable disease in Israel since 2001. Results: The number of reported cases increased during the years 2001-2007, from 775 to 2017.Accordingly, the incidence raised from 12 to 28 per 100,000 persons per year. In 2008 the number of reported cases has decreased. The majority of cases notified (52%-71%) were children aged 1-9 years. The highest incidence rate was found in two Bedouin settlements in the Negev. The disease shows seasonal pattern peaked at the summer. The existence of Giardia cysts in surface water has been monitored in several places in the north since 2000. A possible correlation between the numbers of cysts found in the water to the incidence rates will be discussed.

Prevalence and correlates of *Giardia lamblia* carriage in young Israeli Arab children

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Background: Infection with *Giardia lamblia* ranges between asymptomatic carriage of the organism to a self-limited clinical disease characterized by diarrhea, abdominal cramps temporary weight loss, and malabsorption. *G. lamblia* has a very low infectious dose enabling various modes of transmission and is involved in both sporadic and epidemic morbidity.

Objectives: To determine the prevalence and correlates of *G. lamblia* carriage in healthy young Israeli Arab children, a population under socio-economic and environmental transition

Methods: We conducted two studies, in 2003-2004 and in 2007-2008, among healthy children from the villages Jeser El-Zarka, Faradis and Kfar-Qaraa. Stool specimens were collected from the participants and examined for *G. lamblia* by direct light microscopy of saline mount preparations. Parents were interviewed on demographic, socioeconomic and environmental data.

Results: In the 2003-2004 study, 226 children aged 3-5 years, who attended kindergartens, were enrolled. The carriage rate of *G. lamblia* was 16.8% (95%CI 12.5 - 22.2). It was highest in Jeser El-Zarka (29.5%) compared with 6.3% and 8.8% in Kfar Qaraa and Faradis, respectively ($p<0.001$). Carriage of *G. lamblia* was significantly associated with living in crowding conditions, low parental education, low income, number of siblings, contact with domestic animals, lack of kitchen cupboard and contact between houseflies and foods in the households. The correlates maintaining association in the multivariate analysis were living in crowding conditions: OR 3.8 (95%CI 1.3-10.5), low parental education: OR 7.0 (95% CI 0.76-65.5), and lack of kitchen cupboard OR 3.9 (95%CI 1.3-11.8). *G. lamblia* carriage was not associated with nutritional status. A sub-sample of this cohort was re-examined for *G. lamblia* 4 years later (at ages 7-9) yielding a *G. lamblia* infection rate of 6.7% (95%CI 3.7 - 11.8). In the 2007-2008 study, a cohort of 252 newborns from the villages Faradis and Jeser El Zarka were recruited and monitored for enteric infections during the first 18 months of life by biweekly follow up interviews and examination of stool samples for carriage of enteropathogens at selected ages. The carriage rates of *G. lamblia* were 0%, 2.3%, 1.7%, 6.0% and 18.2% at 2-4, 6, 8, 12 and 18 months of age, respectively.

Conclusions: *G. lamblia* carriage rate was the highest in children aged 3-5 years who attended kindergartens. Low socioeconomic characteristics were the main risk factors of *G. lamblia* infection in the sample of Israeli Arab children studied.

Immune response to *Giardia lamblia* infection in infancy

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Infection with *Giardia lamblia* varies in both its severity and duration with most infected children remaining asymptomatic. A high incidence of giardiasis is observed in immunosuppressed subjects, specifically in those who are immunoglobulin-deficient. The humoral and cellular immune responses to the parasite were studied in two populations of infants and young children; children attending a day-care center in whom Giardia infection was asymptomatic and children residing in a rural setting, in close proximity to farm animals, in whom exposure to the parasite occurs at a very early age, prevalence of infestation is high and infection is associated with diarrheal episodes. In day –care

children, infection, albeit asymptomatic, was accompanied by a significant increase in anti-Giardia IgM levels. In contrast, in the rural population group, levels of specific antibodies of IgA, IgM and IgG classes were comparable in children with and without Giardia- associated diarrheal disease and were up to five hold higher than levels in day –care children. In asymptomatic day –care children specific salivary antigiardia IgA antibodies, measured using an ELISA assay developed in our lab, showed higher levels in infected children with a significant rise in OD levels when, based upon serial stool tests, infestation first occurred. Peripheral blood lymphocyte subsets of rural population children with either acute gastroenteritis, *G. lamblia*-associated diarrhea or without diarrhea were compared. Proportion of CD8 and CD57 (NK+ subset of CD8))cells and of CD4CD29 ("memory" helper T cells) was highest in infants with acute gastroenteritis, lower in infants without diarrhea and lowest in infants with Giardia-associated diarrhea. Thus, in contrast to acute gastroenteritis from other causes *G. lamblia*-associated diarrhea did not elicit changes in lymphocyte subsets. Further studies in an older group of children, from a similar population, failed to show differences in PBL between children with Giardia-negative or Giardia-positive, recurrent diarrheal episodes.

Parasitic infection as a cause of chronic diarrhea in returning travelers

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Diarrhea and gastro-intestinal (GI) symptoms are the most common complaints in returned travelers, accounting for about 40% of all post travel referrals. Among the GI complaints persistent or chronic diarrhea is the most common reason for seeking medical care. In this scenario bacteria are uncommon and parasite infections are more likely to explain the symptoms. The most common protozoan pathogen isolated in travelers with persistent diarrheal symptoms are Giardia lamblia, followed by E. histolytica. However, in only 29% of our patients there were any positive findings of pathogens in the stools, while the etiology agent of the diarrhea in the remaining 71% of patients could not be established. Accordingly, in most cases of post travel chronic diarrhea no pathogen could be recovered. it is not clear whether this high percentage of negative results should be attributed to pathogens that are not recovered due to the low sensitivity of the tests currently available, to yet unrecognized pathogens, or to a non-infectious illness. Many patients with persistent travelers' diarrhea where no other cause is found are diagnosed as having post-infectious irritable bowel syndrome (PI-IBS). Since several protozoa infections may cause IBS-like symptoms, and due to the fact that sensitivity of stool tests is far from being optimal, we recommend an empiric trial of broad spectrum anti-parasitic treatment, before entitle the patient with IBS diagnosis. Further investigation is warranted for the evaluation of the etiology, treatment and the long-term prognosis of post travel chronic diarrhea.

Allicin from garlic – an ancient remedy for infectious diseases revisited

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Garlic is perhaps the oldest medicinal plant which has been used by Man to cure and treat a variety of maladies. Ancient civilizations independently discovered its potent antimicrobial effects and the Chinese still use an alcohol extract, from crushed cloves, to treat patients suffering from diarrheas and respiratory infections. The biologically active principle of Garlic is the molecule Allicin which is produced by the enzyme Alliinase from the inert substrate Alliin following the crushing of a Garlic clove. Pure Allicin molecules are produced in our lab by passing a solution of synthetic, Nature-identical Alliin through a column containing immobilized Alliinase. Allicin is a heat-sensitive, slightly volatile molecule which is responsible for the typical smell of freshly crushed Garlic. Allicin is a hydrophobic molecule that easily penetrates membranes of cells where it reacts by thiolation of free thiol groups and inhibits, at micromolar concentrations, the activity of a variety of enzymes causing the rapid death of a wide range of microorganisms. Pure Allicin was shown to have a potent antimicrobial effect on a variety of protozoan parasites which infect millions of people around the world such as *Entamoeba, Giardia, Plasmodium, Trichomonas, Leishmania* and *Trypanosomes*. Allicin is also very potent against a variety of fungal pathogens such as *Aspergillus, Candid*a and *Onchomycetes*. Allicin, at higher concentrations (20 µM), can also kill mammalian cells and a system for the in-vivo delivery and production of toxic Allicin molecules at the site of a specific target cell, such as a Cancer or Fungal cell, is in the process of development.

POSTERS

The importance of routine culture media in the detection of amoebiasis

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In 1971, we published the principles of preparation of our routine culture medium in Isr. J. Med. Sciences. As a witness of its usefulness is the letter of Dr. K. Ozgur from Turkey, in which he informed us that he used this culture medium and obtained excellent results. The culture medium is used in coprology because it enables the few and rare protozoa excreted, to multiply. This culture medium was used in growing trophozoites for preparing *Entamoeba histolytica* antigens used in serology for IHT for amoebiasis and because it does not contain any serum components, there are no interferences of Forsman complexes. The culture medium is used in maintaining control strains of *E. histolytica* isolate in local cases. It is easy to prepare, no expensive and available in commercial kits.